

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

A327 CT USA

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/914175

INTERNATIONAL APPLICATION NO.

PCT/US00/04744

INTERNATIONAL FILING DATE

25 February 2000

PRIORITY DATE CLAIMED

26 February 1999

TITLE OF INVENTION

BONE MARROW TRANSPLANTATION FOR HEPATIC REGENERATION AND REPAIR

APPLICANT(S) FOR DO/EO/US

PETERSEN, Bryon E. and GOFF, Julie P.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Form PCT/IB/301; Form PCT/IB/304; Form PCT/IPEA/401; Form PCT/IPEA/402; Form PCT/IB/308; From PCT/IB/332; Form PCT/IPEA/408; response to Written Opinion; Amendment Under Article 34; a postcard and a check in the amount of \$501.

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

INTERNATIONAL APPLICATION NO.

ATTORNEY'S DOCKET NUMBER

09/914175

PCT/US00/04744

A32212-PCT USA

24. The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00
- ☒ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =**\$690.00**

Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).

☐ 20 ☐ 30**\$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	24 - 20 =	4	x \$18.00
Independent claims	6 - 3 =	3	x \$80.00

\$72.00**\$240.00**

Multiple Dependent Claims (check if applicable).

☐**\$0.00****TOTAL OF ABOVE CALCULATIONS =****\$1,002.00**

- ☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.

\$501.00**SUBTOTAL =****\$501.00**

Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).

☐ 20 ☐ 30

+

\$0.00**TOTAL NATIONAL FEE =****\$501.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

☐**\$0.00****TOTAL FEES ENCLOSED =****\$501.00**Amount to be:
refunded

\$

charged

\$

- ☒ A check in the amount of **\$501.00** to cover the above fees is enclosed.
- ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-4377**. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Rochelle K. Seide, Ph.D.
BAKER BOTTS LLP
30 Rockefeller Plaza
New York, NY 10112-0228

SIGNATURE

Carmella L. Stephen

NAME

41,328

REGISTRATION NUMBER

24 August 2001

DATE



Filed PCT/PTO 01 MAR 2002

FILE NO. A32212-PCT-USA 072396.0234
PATENT

#5/a

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Petersen et al.
Serial No. : 09/914,175 Examiner : TBD
Filed : August 24, 2001 Group Art Unit: TBD
For : BONE MARROW TRANSPLANT FOR HEPATIC REGENERATION
AND REPAIR

RESPONSE TO NOTIFICATION OF MISSING
REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED
STATES DESIGNATED/ELECTED OFFICE

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

February 20, 2002

Date of Deposit

Carmella L. Stephens

Attorney Name

41,328

PTO Registration No

Carmella L. Stephens
Signature

February 20, 2002

Date of Signature

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notice of Missing Requirements Under 35 U.S.C. 371 in the United States Designated/Elected Office mailed on November 20, 2001, Applicants submit the following amendment and remarks. In accordance with 37 C.F.R. §1.821-1.825, Applicants submit herewith an initial Sequence Listing in computer and paper form. Applicants also request

a one month extension of time for response under 37 C.F.R. 1.136 (a) and enclose the required fee pursuant to 37 C.F.R. 1.17 (a)(1).

IN THE SPECIFICATION

Please **amend** the paragraph beginning on page 20, line 20 and ending on page 21, line 2 as follows:

PCR analysis for the Y chromosome was performed on DNA extracted from transplanted and non-transplanted female animals using primers for the *sry* gene of the Y chromosome (An, J. et al., 1997, J Andrology 18:289). The primer sequences are as follows:

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5'-ATAGTGTGTAGGTTGTTGTCC-3' (SEQ ID NO. 2). These primers amplify a 549-bp

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Alto, CA), included to ensure that each sample of DNA was intact, produce an expected product of approximately 1000 base pairs in length (DeFrances, M.C., 1992, Development 116:387).

REMARKS

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Attached hereto is a marked-up version of the changes made to the specification.

The attached page is captioned "VERSION WITH MARKINGS TO SHOW CHANGES
MADE" and is only included for the Examiner's convenience. Should any discrepancies be
discovered, the version presented in the preceding "IN THE SPECIFICATION" section shall
take precedence.

CONCLUSION

Please charge any additional fees associated with this filing or credit any
overpayment to Deposit Account No. 02-4377. Two copies of this paper are enclosed.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

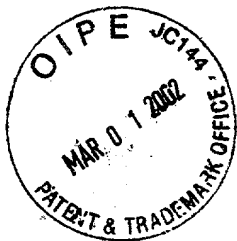
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Rec'd PCT/PTO 01 MAR 2002
FILE NO. A32212-PCT-USA 072396.0234
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February 20, 2002

Date of Deposit

Carmella L. Stephens

Attorney Name

41,328

PTO Registration No

Carmella L. Stephens
Signature

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Assistant Commissioner for Patents
Washington, D.C. 20231

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SEQUENCE LISTING

<110> Bryon E. Petersen
Julie P. Goff

<120> BONE MARROW TRANSPLANT FOR HEPATIC
REGENERATION AND REPAIR

<130> A32212-PCT-USA 072396.0234

<140> 09/914,175

<141> 2001-08-24

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201403041650

BONE MARROW TRANSPLANTATION FOR HEPATIC REGENERATION AND REPAIR

SPECIFICATION

1. INTRODUCTION

5 The present invention relates to methods and compositions for stimulating liver regeneration in subjects with liver disorders. Specifically, the methods and compositions of the invention provide for the transplantation of bone marrow cells into a recipient host in amounts sufficient to result in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration. The invention is
10 based in the observation that bone-marrow derived cells, can participate in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration.

 The present invention further provides methods for deriving enriched populations of hepatic oval cells, considered to be hepatic stem cells, utilizing antibodies that recognizes the Thy-1 cell surface antigen expressed on the surface of
15 hepatic oval cells. The enriched populations of hepatic oval cells can be transplanted into a host for stimulating liver regeneration in subjects with liver disorders. The present invention, by enabling methods for the transplantation of bone marrow cells and/or oval cells for stimulation of liver regeneration provides a safer alternative to whole liver transplantation in subjects having liver disorders including, but not limited
20 to, cirrhosis of the liver, alcohol induced hepatitis, chronic hepatitis, primary sclerosing cholangitis and alpha₁-antitrypsin deficiency.

2. BACKGROUND OF INVENTION

 The origin of the hepatic oval cell has been a topic of considerable interest and controversy for the past several decades. Because oval cells proliferate
25 when hepatocytes are prevented from proliferating in response to liver damage, these cells have been considered to be hepatic stem cells, or the intermediate progeny of a hepatic stem cell. The prevailing opinion is that oval cells originate either from cells

present in the canals of Herring (Grisham, J.W. and Thorgiersson, S.S. in Stem Cells, C.S. Potter, Ed. (Academic Press, San Diego, CA 1997) pp. 233-282) or from blast-like cells located next to bile ducts (Novikoff, P.M. et al., 1996, Am J. Pathol. 148:1473). Oval cells are not easily detected in normal livers. In certain pathological conditions, however, in which an inhibition of hepatocyte proliferation is followed by severe hepatic injury, oval cells are readily apparent due to their active proliferation. In recognized experimental animal models, hepatocyte proliferation is generally suppressed by exposure of the animal to 2-acetylaminofluorene (2-AAF) and, subsequently, hepatic injury is usually induced by partial hepatectomy (PHx) or by administration of carbon tetrachloride (CCl₄) (Evarts, R.P., et al., 1989, Cancer Res. 49:1541; Petersen, B.E. et al., 1998, Hepatology 27:1030). Oval cells have been observed in organs other than the liver, such as the pancreas of rats fed a copper-deficient diet (Bartles, J.R. et al., 1991, J Cell Science 98:45; Rao, M.S. and Reddy, J.K., 1995, Seminars in Cell Biology 6:151).

Throughout life, hepatic and hematopoietic cells intermingle and appear to be interdependent. During fetal life, hematopoietic stem cells (HSC) move out of the yolk sac and take up residency in the liver, and until the time of birth, the liver functions as a hematopoietic organ (Baker, J.E. et al., 1969, J Cell Physiol 74:51; Moore, M.S. et al., 1970, Br J Haematol 18:279). This function ceases in the neonate, but under certain conditions it can be reactivated in the form of extra-medullary hematopoiesis (Tsamanda, A.C., 1995, Modern Pathol 8:671). The adult liver has been shown to harbor a significant number of HSC (Hayes, E.F. et al., 1975, J Cell Physiol 86:213), and it has been shown that the bone marrow of lethally irradiated animals can be reconstituted by whole liver transplantation (Murase N et al., 1996 Transplantation 61:1).

Hematopoietic activity and erythropoietic cells have been shown to reappear in the liver during liver regeneration following a partial hepatectomy (Naughton, B.A. et al., 1982, Exp Hematol 10:451; Barbera-Guillem, E. et al., 1989 Hepatology 9:29; Sakamoto, T., et al, 1992, Reg Immunol 4:1). It has also been shown that cultured rat hepatocytes can produce granulocyte-macrophage colony stimulating factor, a known hematopoietic cytokine (Sakamoto, T. et al., 1991, Reg

Immunol 3:260). Recently, oval cells were found to express CD34, Thy-1 and c-kit mRNAs and proteins (Petersen, B. et al., 1998, Hepatology 27:433; Omori, N. et al, 1997, Hepatology 26:720; Fujio, K. et al., 1994, Lab Invest 70:511). These antigens are known to be expressed on HSC. In addition, oval cells express *flt-3* receptor mRNA, which in humans and mice has been reported to be restricted to populations of hematopoietic progenitor cells (Omori, M. et al., 1997, Am J Pathol 150:1179). The adult mammalian liver can therefore be considered a potential hematopoietic organ.

3. SUMMARY OF THE INVENTION

The present invention provides methods and compositions for stimulating liver regeneration in subjects with liver disorders. The compositions and methods of the invention provide for the transplantation of bone marrow cells into a recipient in amounts sufficient to result in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration. The invention is based on the observation that bone-marrow derived cells, can participate in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration.

The present invention further provides methods for deriving enriched populations of hepatic oval cells, considered to be hepatic stem cells, utilizing antibodies that recognizes the Thy-1 cell surface antigen expressed on the surface of hepatic oval cells. The enriched populations of hepatic oval cells may also be utilized in methods directed to regeneration of liver tissue.

The present invention also provides compositions for use in stimulating liver regeneration comprising bone marrow cells and/or hepatic oval cells in a pharmaceutical acceptable carrier. The compositions of the invention may be utilized for treatment of subjects with liver disorders where the stimulation of liver regeneration is desired. Such disorders include cirrhosis of the liver, alcohol-induced hepatitis, chronic hepatitis, primary sclerosing cholangitis, alpha₁-antitrypsin deficiency and liver cancer.

In an embodiment of the invention, the bone marrow derived stem cells and/or hepatic oval cells may be genetically engineered, prior to transplantation, to enable them to produce a wide range of proteins, including but not limited to, growth

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factors, cytokines, or biologically active molecules, such as hormones. In this way, any new liver tissue derived from the transplanted stem cells or hepatic oval cells will produce the desired biologically active protein.

The invention further relates to the *in vitro* attachment of stem cells or hepatic oval cells to a matrix prior to transplantation for the purpose of increasing the viability and growth of the transplanted cells. In addition, the matrix may be composed of additional materials including other types of cells or biologically active molecules.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. PCR analysis of DNA from female rats transplanted with bone marrow from the femurs of male donors rats. The data presented here show that the transplanted animals tested positive for the Y chromosome in both the Thy1.^l and Thy1.^l sub-populations of non-parenchymal cells (NPC) at both days tested. The Thy-1^l fraction shows strong signal probably due to the presence of hematopoietic cells. After successful BMTx, presumably the mature (differentiated) male hematopoietic cells could be found in the NPC population. The mature hematopoietic (i.e. Kupffer cells, monocytes, etc.) cells will be negative for Thy-1, but positive for the Y chromosome. Note the Day 13 fraction of hepatocytes are now expressing the Y chromosome PCR product. This is the time point when oval cells begin to make the transition to hepatocytes. As noted in the text, the Day 9 hepatocyte fraction was negative for the Y chromosome. This would be expected in the prevailing view of the timing events in the cascade of oval cell proliferation and differentiation. The control female DNA as well as the non transplanted female DNA is negative for Y chromosome expression. The β -actin product reveals that the DNA was present and intact.

FIGURE 2 A-C. Photomicrographs of *in situ* hybridizations of the Y chromosome *sry* gene performed on frozen liver sections. The arrows indicate positive reaction in the nucleus of hepatocytes. A, untreated control normal male rat (positive control); B, female treated with BMTx and the 2-AFF/CC1₄ protocol and sacrificed at day 13 following hepatic injury; and C, untreated female (negative

control). It should be noted that the color reaction-time for the untreated female was 45 minute, whereas the color reaction-time for both the male and the BMTx/2-AAF/CCl₄ treated female was only 5 minutes. The magnification of all the photomicrographs is 1000X.

5 FIGURE 3 A-F. DPPIV activity in frozen liver sections. A, untreated DPPIV⁺ rat used as a positive control. A diffuse decoration of the bile ductular site of hepatocytes is evident (orange color). B, untreated DPPIV⁻ rat showing a complete absence of DPPIV activity (negative control). C-F are four different BMTx (male DPPIV⁺ donor) DPPIV⁻ rat (recipient) exposed to the 2-AAF/CCl₄ protocol for oval cell induction and sacrificed at day 11 or day 13 following hepatic injury. A positive reaction is evident not only between hepatocytes from all four animals, but also a on a few oval or transitional cells (D and E). The DPPIV staining can appear as a line (open arrowheads) or as a dot (closed arrowheads) depending the plane of the section through the bile cannilcuar region. In all cases there are clusters of cells (2-5) expressing the DPPIV marker. Hepatocytes that are from donor origins are denoted by *. Original magnifications, 200x.

20 FIGURE 4 A-B. Sections from two Brown-Norway rat livers, transplanted into Lewis recipient rats, sacrificed 11 days (A) and 13 days (B) after CCl₄ administration in the oval cell induction protocol. The sections were immunostained with an L21-6 mAb. Positive oval cells (arrow heads) and positive cells in ductal structures (arrows) can be seen. Original magnifications, A, 100x, and B, 200x.

25 FIGURE 5 A-D. Frozen liver section showing double immunofluorescence staining of a periportal region in a Brown-Norway liver transplanted into a Lewis recipient rat (A). Green fluorescence: anti L21-6, a recipient marker; red fluorescence: anti OC-2, an oval cell and a mature ductal cell marker. When the two antibodies are in close proximity, the light waves mix and the emitted fluorescence is yellow. Oval cells co-expressing the two markers are evident (arrows). Other cells can be seen that express only L21-6 (presumably inflammatory cells), or only OC.2 (oval cells that could have been derived from, L21-6 negative precursor-cells resident in the Brown-Norway liver). B-D shows a frozen section of a

Brown-Norway liver transplanted into a Lewis recipient rat and put on the 2-AAF/ CCl_4 protocol, day 13 after CCl_4 exposure. Centered in the photomicrograph is a ductal structure (B-D), the same type of structure seen in Figure 4A. Double immunofluorescence staining was performed using anti-L21-6 (B) and anti-OC-2 (D). Merged images of the L21-6 and OC-2 immunofluorescence staining are shown in the middle photomicrograph (C). Cells expressing both antigens are yellow (C). As a point of origin, * indicates the center of the bile duct. The origin of the cells within this duct is extrahepatic because they are positive for L21-6, which makes them recipient derived cells. Arrows in B-D indicate the same cell in all three photomicrographs. Bars represent 10 μm in length.

FIGURE 6. Time line of events for activation of oval cell proliferation. The presence of 2-AAF is necessary to suppress hepatocyte proliferation and to allow extended proliferation of oval cells. The diagram represents the different stages of oval cell proliferation.

FIGURE 7 A-B. Liver section obtained from a rat on day 11 on the 2-AAF/ CCl_4 protocol. Sections were stained with hematoxylin-eosin. Centered in the photomicrograph is a portal triad (PT). The small oval cells (*arrows*) can be seen between the larger hepatocytes. (Original magnification [A] X100; [B] X200.)

FIGURE 8 A-D. Immunohistochemistry for BrdU incorporation on liver sections obtained from animals on the 2-AAF/ CCl_4 protocol at various time points post-hepatic injury. (A) Day 9; (B) day 11; (C) day 13; and (D) positive control for BrdU at the 24-hour Phx time point. The peak of proliferation occurs at day 9, with a drastic drop-off of labeling on subsequent days. The day 13 time point is also represented in Fig. 8. HV, hepatic vein. (Original magnification [A-D] X200.)

FIGURE 9 A-B. Frozen liver sections obtained from normal rat liver. Histological appearance of liver section stained with (A) Thy1.1 antibody and (B) OC.2 antibody. Normal rat liver was used as a negative control for immunostaining procedures. Thy1.1 expression cannot be detected in a normal adult liver, and OC.2 can be detected only on the ductular epithelium in the portal triads. The portal triad region (PT) and central vein region (CV) are designated in the lobule. (Original magnification X40.)

FIGURE 10 A-F. Frozen serial sections from rat liver exposed to the 2-AAF/ CCl_4 protocol (day 21). (A and D) Immunohistochemical expression of CK-19. (B and E) expression of OC.2. (C and F) Expression of Thy1.1 staining as can be seen at lower magnification (A-C), CK-19, OC.2, and Thy1.1 exhibit nearly the same staining pattern. At higher magnification (D-F), the individual oval cells stain positive for each antibody. Open *arrows* in (A-C) indicate the reference point at which the photomicrographs of higher magnifications were taken. *Solid arrows* in (D-F) show individual oval cells positively stained for the appropriate antibody. It should be noted that the ductular epithelium is now staining positive for Thy-1. In normal liver, this is not the case. This same result can be seen in Fig. 6 as well. (Original magnification [A-C] X40; [D-F] X200.)

FIGURE 11 A-F. Frozen serial sections from rat liver exposed to 2-AAF/PHx protocol (day 13). (A and D) Immunohistochemical expression of CK-19. (B and E) Expression of OC.2. (C and F) Expression of Thy1.1 staining. At lower magnification (A-C), CK-19, OC.2, and Thy1.1 exhibit nearly the same staining pattern. At higher magnification (D-F), the individual oval cells stain positive for each antibody. *Open arrows* in (A-C) indicate the reference point at which the photomicrographs of higher magnifications were taken. *Solid arrows* in (D-F) show individual oval cells positively stained for the appropriate antibody. This figure illustrates that, in this model, there are no inflammatory cells to confound the issue as to which cell type is expressing Thy-1. Staining patterns are alike for all three markers, and this pattern is similar to what is seen in the 2-AAF/ CCl_4 model. (Original magnification [A-C] X40; [D-F] X200.)

FIGURE 12 A-D. Profiles of NPC fraction obtained from perfused rat liver exposed to the 2-AAF/ CCl_4 protocol. Cells were labeled with Thy1.1-FITC antibody and sorted by flow cytometry. Cells were separated into two factions: (A) right gate, Thy-1⁺-labeled cells and (B) left gate, Thy-1⁻ cells. Histograms revealed that 95% to 97% purity could be obtained for Thy-1.1⁺ cells, while 99% purity could be achieved for negative cells. (C) Sorted Thy1.1⁺ cells were stained with PI, and flow cytometric cell cycle analysis was performed. Analysis revealed that 94% Thy1.1⁺ cells are in the G0/G1 (resting) stage of the cell cycle. (D) Thy-1-labeled

cells (*dark solid line*) that are in the G0/G1 stage of the cell cycle (*dashed line*). Approximately 90% of the resting cells are Thy-1⁺.

FIGURE 13 A-B. Immunohistochemistry of liver section obtained from a day 13 2-AAF/CCl₄ rat. (A) BrdU staining of oval cells in the proliferative state. Very few cells are positively stained, which may represent the small peak of cells in the G2/M phase seen in Fig. 7C and 7D. The ductular formation (BD) is devoid of any BrdU staining. (B) Liver section stained for PCNA also shows little or no PCNA staining. This corroborates the BrdU staining. Both of these stains document that the majority of oval cells taken at this time point are not in a proliferative state. © and D) The corresponding positive controls for BrdU and PCNA staining on rat liver sections from the 24-hour Phx time point. *Arrows* indicate individual oval cells. (Original magnification [A-D] X100.)

FIGURE 14 A-H. Cytocentrifuged preparation of Thy1.1⁺ sorted cells from 2-AAF/CCl₄-treated rats. Thy-1.1⁺ were stained with oval cell-specific antibodies. (A) Hematoxylin-eosin stain. (B) A representative of negative control (omission of primary antibody). (C-F) AFP, CK-19, GGT, and OV6 staining, respectively. The majority of the Thy-1⁺ cells were positive for oval cell-specific markers. (G) A representative of cells stained for desmin. All photomicrographs are at 100X magnification. (H) Dual staining of oval cells. AFP-Texas Red (*red*) and Thy1.1-FITC (*green*) showing both markers on the same cells. Where the two antibodies are in close proximity to each other, the wavelengths mix and the resulting fluorescence is yellow. Photomicrograph for (H) was obtained using confocal microscopy.

FIGURE 15 A-E. Cytocentrifuged preparation of Thy1.1⁺ sorted cells from 2-AAF/CCl₄-treated rats. The Thy-1⁺ cells were also stained with oval cell antibodies. (A) Hematoxylin-eosin stain. (B and D) Cells stained for AFP and GGT, respectively. The corresponding negative controls are shown in (C) and (E). All photomicrographs are at 200X magnification.

FIGURE 16 A-F. (16A) Normal Rat Spleen. DPPIV positive cells with stain with a reddish/burn orange color. Notice that the white pulp region of the spleen is devoid of stain. 10x objective. (16B) Normal Rat Spleen. DPPIV positive

cells with stain with a reddish/burn orange color. Notice that the white pulp region of the spleen is devoid of stain. 20x objective. (16C) Experimental DPPIV deficient rat transplanted with DPPIV positive bone marrow. Spleen 60 days post BMTx. Notice the reddish/burn orange staining present throughout the spleen. The white pulp region is now showing a large number of cells positive for DPPIV. 4x objective. (16D) Experimental DPPIV deficient rat transplanted with DPPIV positive bone marrow. Spleen 60 days post BMTx. Notice the reddish/burn orange staining present throughout the spleen. The white pulp region is now showing a large number of cells positive for DPPIV. 10x objective.

FIGURE 17A-D. (17A) Normal Rat pancreas. DPPIV positive cells with stain with a reddish/burn orange color. 10x objective. (17B) Normal Rat pancreas. DPPIV positive cells with stain with a reddish/burn orange color. 20x objective. (17C) DPPIV deficient rat pancreas. No staining is visible throughout the section. 10x objective. (17D) DPPIV deficient rat pancreas. No staining is visible throughout the section. 20x objective. (17E) Experimental DPPIV deficient rat transplanted with DPPIV positive bone marrow. Pancreas 60 days post BMTx. Notice there are a few reddish/burn orange cells staining positive in the pancreas. 10x objective. (17F) Experimental DPPIV deficient rat transplanted with DPPIV positive bone marrow. Pancreas 60 days post BMTx. Notice there are a few reddish/burn orange positive cells present in the pancreas. 20x objective.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods and compositions for stimulating liver regeneration in a subject having a liver disorder. The invention provides methods and compositions for transplanting of bone marrow cells into a recipient host in amounts sufficient to result in the production of hepatocytes, bile ductal cells and oval cells during liver regeneration.

In a specific embodiment of the invention, bone marrow cells are administered to a subject in need of new liver tissue. The bone marrow cells can be injected into the recipient, wherein the bone marrow cells will migrate to the liver, undergo proliferation, and differentiation leading to the production of new liver tissue

containing hepatocytes, bile ductal cells and oval cells. Alternatively, the bone marrow cells may be transplanted directly into the liver where the cells will proliferate and differentiate to form new liver tissue.

5 The compositions of the invention comprise bone marrow cells in a pharmaceutically acceptable carrier for administration into a recipient host in need of new liver tissue. The bone marrow cells may also be genetically engineered to enable them to produce a wide range of functionally active proteins, such as for example, growth factors, cytokines and hormones. The compositions of the invention also
10 comprise bone marrow cells on a support matrix for transplantation into the liver. The matrix may further comprise growth factors capable of stimulating the proliferation and/or differentiation of hepatic stem cells or other types of cells.

The invention further relates to methods for enriching for populations of hepatic oval cells, a hepatic stem cell, using the Thy-1 cell surface antigen as an antibody tag. Once purified, the oval cells may be transplanted into a recipient in need
15 of new liver tissue. The hepatic oval cells may be transplanted directly into the liver of the recipient where the hepatic oval cells will undergo proliferation, and differentiation leading to the production of new liver tissue containing hepatocytes, bile ductal cells and oval cells. Alternatively, the cells may be injected into the portal vein where the cells will go directly to the liver. In yet another embodiment of
20 the invention, the hepatic oval cells may be injected into the spleen followed by migration to the liver.

5.1. SOURCES OF BONE MARROW CELLS

Bone marrow cells may be obtained from a variety of different donor sources. In a preferred embodiment, autologous bone marrow is obtained from the
25 subject who is to receive the bone marrow cells. This approach is especially advantageous since the immunological rejection of foreign tissue and/or a graft versus host response is avoided. In yet another preferred embodiment of the invention, allogenic bone marrow may be obtained from donors who are genetically related to the recipient and share the same transplantation antigens on the surface of their blood

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cells. Alternatively, if a related donor is unavailable, bone marrow from antigenically matched (identified through a national registry) donors may be used.

Bone marrow cells can be obtained from the donor by standard bone marrow aspiration techniques known in the art. For example, bone marrow cells can be removed from the donor by placing a hollow needle into the marrow space and withdrawing a quantity of marrow cells by aspiration. Alternatively, peripheral stem cells can be obtained from a donor, for example, by standard phlebotomy or apheresis techniques. For convenience, the following embodiments of the invention are described for bone marrow cells, although it should be understood that peripheral stem cells may be used as equivalent to bone marrow cells.

Before administration into the recipient, bone marrow cell populations may be enriched for stem cells by selecting for cells that express stem cell surface antigens such as Thy-1, CD34, *Flt-3* ligand and *c-kit*, in combination with purification techniques such as immuno-magnetic bead purification, affinity chromatography and fluorescence activated cell sorting. In addition, where the possibility of a graft versus host response exists, the stem cells to be administered to the recipient can be T-cell depleted to prevent the development of a graft versus host response. The cell population may be depleted of T-cells by one of many methods known to one skilled in the art (e.g., Blazer et al., 1985, *Experimental Hematology* 13:123-128).

Prior to transplantation into the recipient host, the bone marrow cells may be stimulated with a number of different growth factors that can regulate tissue regeneration by affecting cell proliferation, differentiation and gene expression. Such growth factors include those capable of stimulating the proliferation and/or differentiation of bone marrow cells and hepatic progenitor cells. For example, epidermal growth factor (EGF), transforming growth factor α (TGF- α) or hepatocyte growth factor/scatter factor (HGF/SF) may be utilized. The bone marrow cells may be stimulated *in vitro* prior to transplantation into the recipient subject. Alternatively, the stem cells may be stimulated *in vivo* by injecting the recipient with such growth factors following transplantation.

The present methods and compositions can also employ bone marrow cells genetically engineered to enable them to produce a wide range of functionally

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active biologically active proteins, including but not limited to growth factors, cytokines, hormones, inhibitors of cytokines, peptide growth and differentiation factors. Methods which are well known to those skilled in the art can be used to construct expression vectors containing a nucleic acid encoding the protein coding region of interest operatively linked to appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook, et al., 1992, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., and Ausebel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.

In addition, stem cells may be attached *in vitro* to a natural or synthetic matrix that provides support for the transplanted cells prior to transplantation. The type of matrix that may be used in the practice of the invention is virtually limitless. The matrix will have all the features commonly associated with being "biocompatible", in that it is in a form that does not produce an adverse, or allergic reaction when administered to the recipient host. Growth factors capable of stimulating the growth and regeneration of liver tissue may also be incorporated into the matrices. Such matrices may be formed from both natural or synthetic materials and may be designed to allow for sustained release of growth factors over prolonged periods of time. Thus, appropriate matrices will both provide growth factors and also act as an *in situ* scaffolding in which the transplanted cells differentiate and proliferate to form new liver tissue. In preferred embodiments, it is contemplated that a biodegradable matrix that is capable of being reabsorbed into the body will likely be most useful.

To improve stem cell adhesion to the matrix, and survival and function of the stem cell, the matrix may optionally be coated on its external surface with factors known in the art to promote cell adhesion, growth or survival. Such factors include cell adhesion molecules, extra cellular matrix molecules and/or growth factors.

The present invention also relates to the use of bone marrow cells in three dimensional cell and tissue culture systems to form structures analogous to liver tissue counterparts *in vivo*. Cells cultured on a three-dimensional culture system will

grow in multiple layers, forming new liver tissue. The resulting liver tissue will survive for prolonged periods of time, and perform liver-specific functions following transplantation into the recipient host. Methods for producing such structures is described, *e.g.*, in US Patent No. 5,624,840, which is incorporated herein in its entirety.

5.2 METHODS FOR ENRICHMENT OF HEPATIC OVAL CELLS

The present invention also provides methods for deriving an enriched population of hepatic oval cells from liver tissue using a Thy-1 specific antibody. This aspect of the invention is based on the observation that hepatic oval cells express high levels of Thy-1 on their cell surface.

Hepatic oval cells may be obtained from a variety of different donor sources. Depending on the degree of liver damage, enriched populations of autologous hepatic oval cells may be derived from the tissue of the subject who is to receive the transplanted hepatic oval cells. This approach avoids the immunological rejection of foreign tissue. In yet another preferred embodiment of the invention, allogenic liver tissue for use in purifying hepatic oval cells may be obtained from donors who are genetically related to the recipient and share the same transplantation antigens on the surface of their blood cells. Alternatively, if a sibling is unavailable, tissue may be derived from antigenically matched (identified through a national registry) donors.

In an embodiment of the invention, hepatic oval cells are isolated from a disaggregated liver tissue biopsy. This may be readily accomplished using techniques known to those skilled in the art. For example, the liver tissue can be disaggregated mechanically and/or treated with digestive enzymes and/or chelating agents that weaken the connections between neighboring cells, making it possible to disperse the tissue suspension of individual cells. Enzymatic dissociation can be carried out by mincing the liver tissue and treating the minced tissue with any of a number of digestive enzymes. Such enzymes include, but are not limited to, trypsin, chymotrypsin, collagenase, elastase and/or hyaluronidase. A review of tissue disaggregation technique is provided in, *e.g.*, Freshney, Culture of Animal Cells, A

Manual of Basic Technique, 2d Ed., A.R. Liss, Inc., New York, 1987, Ch. 9, pp.107-126.

Following preparation of a single cell suspension, Thy 1.1-positive cells, which represent the hepatic oval cell population of cells may be purified from the Thy1-1-negative population of cells using a variety of different methods. Such procedures involve a positive selection, such as passage of sample cells over a column containing anti-Thy-1 antibodies or binding of cells to magnetic bead conjugated anti-Thy1 antibodies or by panning on anti-Thy-1 antibody coated plates and collecting the bound cells. Alternatively, the single cell suspension may be exposed to a labeled antibody that immuno-specifically binds to the Thy1-1 cell surface antigen. Following incubation, with the Thy 1.1 antibody, the cells are rinsed in buffer to remove any unbound antibody. Hepatic oval cells expressing the Thy1-1 cell surface antigen can then be cell sorted by fluorescence-activated cell sorting using, for example, a Becton Dickinson FACStar flow cytometer.

Prior to transplantation into the recipient host, the hepatic oval cells may be contacted with a number of different growth factors that can regulate tissue regeneration by affecting cell proliferation, and gene expression. Such growth factors include those capable of stimulating the proliferation and/or differentiation of hepatic progenitor cells. For example, epidermal growth factor (EGF), transforming growth factor α (TGF- α) or hepatocyte growth factor/scatter factor (HGF/SF) may be utilized. The hepatic oval cells may be stimulated *in vitro* prior to transplantation into the recipient subject, or alternatively, by injecting the recipient with growth factors following transplantation.

The present methods and compositions may employ hepatic oval cells genetically engineered to enable them to produce a wide range of functionally active biologically active proteins including, but not limited to, growth factors, cytokines, hormones, inhibitors of cytokines, peptide growth and differentiation factors. Methods which are well known to those skilled in the art can be used to construct expression vectors containing a nucleic acid encoding the protein of interest linked to appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook, et al., 1992, Molecular Cloning, A Laboratory

Manuel, Cold Spring Harbor Laboratory, N.Y., and Ausebel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.

5 In addition, hepatic oval cells may be attached *in vitro* to a natural or synthetic matrix that provides support for the transplanted hepatic oval cells prior to transplantation. The matrix will have all the features commonly associated with being "biocompatible", in that it is in a form that does not produce an adverse, or allergic reaction when administered to the recipient host. Growth factors capable of stimulating the growth and regeneration of liver tissue may also be incorporated into
10 matrices. Such matrices may be formed from both natural or synthetic materials and may be designed to allow for sustained release of growth factors over prolonged periods of time. Thus, appropriate matrices will both provide growth factors and also act as an *in situ* scaffolding in which the hepatic oval cells differentiate and proliferate to form new liver tissue. In preferred embodiments, it is contemplated that a
15 biodegradable matrix that is capable of being reabsorbed into the body will likely be most useful.

To improve oval cell adhesion to the matrix, and survival and function of the stem cell, the matrix may optionally be coated in its external surface with factors known in the art to promote cell adhesion, growth or survival. Such factors
20 include cell adhesion molecules, extra cellular matrix molecules or growth factors.

The present invention also relates to the use of hepatic oval cells in three dimensional cell and tissue culture systems to form structures analogous to liver tissue counterparts *in vivo*. The resulting liver tissue will survive for prolonged periods of time, and perform liver-specific functions following transplantation into the
25 recipient host. Methods for producing such structures is described in US Patent No. 5624,840, which is incorporated herein in its entirety.

5.3. ADMINISTRATION OF BONE MARROW

STEM CELLS OR HEPATIC OVAL CELLS

The bone marrow cells and/or enriched oval cells can be administered
30 to the recipient in an effective amount to achieve its intended purpose. More

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specifically, an effective amount means an amount sufficient to lead to the development of new liver tissue and restoration of liver function, thereby alleviating the symptoms associated with liver disorders.

5 The number of cells needed to achieve the purposes of the present invention will vary depending on the degree of liver damage and the size, age and weight of the host. For example, the cells are administered in an amount effective to restore liver function. The dose range of cells to be used in the practice of the invention may vary between 10^5 - 10^{10} cells, although the preferable dose of administered cells will be between 10^6 - 10^8 . It may be necessary to use dosages
10 outside these ranges in some cases, as will be apparent to those of skill in the art.

Determination of effective amounts is well within the capability of those skilled in the art. The effective dose may be determined by using a variety of different assays designed to detect restoration of liver function. The progress of the
15 transplant recipient can be determined using assays that include blood tests known as liver function tests. Such liver function tests include assays for alkaline phosphates, alanine transaminase, aspartate transaminase and bilirubin. In addition, recipients can be examined for presence or disappearance of features normally associated with liver disease such as, for example, jaundice, anemia, leukopenia, thrombocytopenia,
20 increased heart rate, and high levels of insulin. Further, imaging tests such as ultrasound, computer assisted tomography (CAT) and magnetic resonance (MR) may be used to assay for liver function.

The bone marrow cells and/or enriched oval cells can be administered to the recipient in one or more physiologically acceptable carriers. Carriers for these
25 cells may include, but are not limited to, solutions of phosphate buffered saline (PBS) containing a mixture of salts in physiologic concentrations. In addition, the cells may be associated with a matrix prior to administration into the recipient host.

In an embodiment of the invention, the bone marrow cells and/or hepatic oval cells can be administered by intravenous infusion. The cells to be
30 injected, are drawn up into a syringe and injected into the recipient host. In such

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instances the cells would be expected to migrate to the recipient's liver where they will differentiate and proliferate to form new liver tissue.

Alternatively, the methods of the present invention encompass administration of the bone marrow cells and/or hepatic oval cells into the recipient so as to become located in the liver. The administration of the stem cells and/or hepatic oval cells, is accomplished by conventional techniques such as injection of cells into the recipient host liver, injection into the portal vein, or surgical transplantation of cells into the recipient host liver. In some instances it may be necessary to administer the stem cells and/or hepatic oval more than once to restore liver function. In addition, growth factors, such as G-CSF, or hormones, may be administered to the recipient prior to and following transplantation for the purpose of priming the recipient's liver and blood to accept the transplanted cells and/or to generate an environment supportive of hepatic cell proliferation.

5.4. USE OF BONE MARROW CELLS FOR REGENERATION OF TISSUE, OTHER THAN LIVER TISSUE

In yet another embodiment of the invention, bone marrow cells may be used for regeneration of tissues other than liver tissue. Specifically, the methods and compositions of the invention provide for the transplantation of bone-marrow stem cells into a recipient host in amounts sufficient to result in tissue regeneration other than liver regeneration. The invention is based on the observation that bone-marrow derived cells can participate in the production of not only liver cells, but pancreatic cells as well.

The methods of the present invention encompass administration of the bone marrow cells into the recipient host so as to become located in the organ or tissue in which regeneration is desired. The administration of the stem cells into the desired region is accomplished by conventional techniques such as injection of cells within the recipient host or surgical transplantation of cells within the recipient host. In some instances it may be necessary to administer the stem cells more than once to restore the desired tissue function.

In a specific embodiment of the invention, bone marrow cells may be transplanted into the pancreas of a recipient subject in need of new pancreatic tissue. Such subjects include those having pancreatic disorders such as acute or chronic pancreatitis or carcinomas of the pancreas. The bone marrow cells can be administered to the recipient in one or more physiologically acceptable carriers, in an effective amount to achieve its intended purpose. More specifically, an effective amount means an amount sufficient to lead to the development of new pancreatic tissue and restoration of pancreatic function, thereby alleviating the symptoms associated with the pancreatic disorders.

6. EXAMPLE: BONE MARROW CELLS PARTICIPATE IN THE PRODUCTION OF HEPATOCYTES, BILE DUCTAL CELLS AND OVAL CELLS DURING LIVER REGENERATION

The purpose of the present study was to test the hypothesis that oval cells and other liver cells may arise from a cell population originating in, or associated with, the bone marrow. This hypothesis was tested by three approaches: i) bone marrow transplantation (BMTx) from male rats into lethally irradiated syngeneic females, and detection of donor cells in the recipients by means of DNA probes to the Y chromosome *sry* region; ii) BMTx from Dipeptidyl peptidase-IV positive (DPPIV⁺) male rats into DPPIV⁻ syngeneic females, and detection of DPPIV-expressing cells in the recipient animals; and iii) whole liver transplantation (WLTx) using Lewis rats that express the L21-6 antigen as recipients, and Brown-Norway (Brown-Norway) rats that do not express this antigen as allogenic donors, in order to confirm that an extra-hepatic source (L21-6⁺ cells) could repopulate the transplanted (L21-6⁻ cells) liver. In conjunction with these approaches, the 2-AAF/CCl₄ protocol was used to induce oval cell activation and proliferation. Hepatocyte proliferation is generally suppressed by exposure of an animal to 2-acetylaminoflourane (2-AAF) and subsequently hepatic injury is induced by administration of carbon tetrachloride. *In situ* hybridization, PCR,

and immunohisto- and cytochemical techniques were used to distinguish donor cells from recipient cells.

6.1. MATERIALS AND METHODS

6.1.1. BONE MARROW AND WHOLE LIVER TRANSPLANTATION

5 Bone marrow transplantation (BMTx) was performed as previously described (Murase, N. et al., 1996, Transplantation 61:1) with minor modifications. In one set of experiments (Set 1, two separate experiments), bone marrow from male F-344 rats was transplanted into lethally irradiated syngeneic female F-344 rats (n = 10 animals). The females were given 10.5 Gy from a ¹³⁷Cesium source (JL Shepherd Mark I), and then rescued by injecting about 60 x 10⁶ male bone marrow cells via the tail vein. After allowing establishment of a chimeric system (about 30-45 days), the animals were tested to see if the donor cells had engrafted. PCR analysis was performed on DNA extracted from the buffy coat of nucleated cells obtained from retinal orbital blood (ROB). All animals survived the transplant procedure, but
10 varying degrees of intensity for chimerism, was evident. Only those animals that expressed a strong signal for the Y chromosome PCR product were placed on the 2-AAF/CCl₄ protocol for oval cell induction. The animals were sacrificed 9-13 days after CCl₄ administration. In a second set of BMTx experiments (Set 2), bone marrow from male F-344 rats expressing the DPPIV enzyme was transplanted into
15 lethally irradiated DPPIV syngeneic female F-344 rats, which were then further treated as above. Ten female rats were successfully transplanted in Set 1, and fourteen female rats in Set 2.

Whole liver transplantation (WLTx) was performed as previously described (Murase, N et al., 1995, Transplantation 60:158). Brown-Norway rats were
25 used as donors of whole liver tissue, and Lewis rats as recipients. The L21-6 monoclonal antibody specific for Lewis rats was used to distinguish immunohistochemically donor cells from recipient cells (Yagihashi et al., 1995, Transplantation Proceedings 27:1519). Once the rats recovered from the WLTx (about two months), the animals were placed on the 2-AAF/CCl₄ protocol for
30 induction of oval cells as described below. In this experiment six Lewis rats were

successfully transplanted; of these, three animals survived until completion of the study.

6.1.2. ANIMALS

5 F-344 male and female rats were obtained from Frederick Laboratories (Frederick, MD) as marrow donors and recipients. DPPIV⁻ female F-344 rats were a gift from Dr. Sanjeev Gupta, Albert Einstein College of Medicine, Bronx, NY. All procedures involving animals were conducted according to institutionally approved protocols.

6.1.3. INDUCTION OF OVAL CELLS

10 2-AAF time-release pellets were prepared (70 mg, 2.5 mg/day release for 28 days) as described by Hixson et al. (Hixson D.C. et al., 1990, Pathobiology 58:65) and inserted subcutaneously in the rats 7 days prior to administration of CCl₄. The animals were sacrificed, and tissue samples were obtained at various time points thereafter. CC1₄ was injected intraperitoneally (i.p) as a single dose of 1.9 ml/kg
15 (1500 mg/kg) b.w. of a 1: 1 (vol/vol) solution in corn oil; this dose was calculated on the basis of the LD₅₀ dose (RJ. Lewis, Sr., Ed., *SAX Dangerous Properties of Industrial Material* (Van Nostrand Reinhold, New York, NY. 1993), pp. 52 and 1149, eighth edition).

6.1.4. PCR ANALYSIS

20 PCR analysis for the Y chromosome was performed on DNA extracted from transplanted and non-transplanted female animals using primers for the *sry* gene of the Y chromosome (An, J. et al., 1997, J Andrology 18:289). The primer sequences are as follows:
5'-CATCGAAGGGTTAAAGTGCCA-3' and
25 5'-ATAGTGTGTAGGTTGTTGTCC-3'. These primers amplify a 549-bp nucleic acid product that has been shown to be very specific for the Y chromosome with no reactivity to female DNA. PCR was performed as previously described (An, J. et al., 1996, Experimental Hematology 24:768). Human β -actin primers from Clontec Laboratories (Palo Alto, CA), included to ensure that each sample of DNA was intact,

produce an expected product of approximately 1000 base pairs in length (DeFrances, M.C., 1992, Development 116:387).

6.1.5. CELL ISOLATION

Hepatocytes and nonparenchymal cells (NPC) were isolated after a 2-
5 step collagenase digestion of the liver according to an established protocol (1996, Methods Cell Biol 13:29) Prior to the isolation procedure, the caudate lobe was surgically removed and divided into halves, one being fixed in 10% buffered formalin, and the other placed in OTC. compound and frozen in cold 2-Methylbutane. The samples were stored at -80°C until paraffin or frozen sections were prepared for
10 routine examination after hematoxylin and eosin (H&E) staining. This tissue served as an internal control for light microscopy and hybridization *in situ*.

Digestion began by blanching the liver to remove the majority of blood cells from the liver, by perfusion with a buffered saline solution (S&M) for 10 min, 10 ml/min at 37°C. Digestion of the liver was accomplished by collagenase digestion
15 (Worthington Biochemical Co., Freehold, NJ; 100 mg/250 ml S&M supplemented with CaCl) for 20 min, at 10 ml/min at 37°C. On completion of the digestion, the liver was removed from the animal, placed into cold S&M, and repeatedly shaken to disrupt the individual cells from the tissue. The resulting cell suspension was passed through nylon mesh and centrifuged 3 times at 50 g for 5 min to separate the
20 hepatocytes from the NPCs. The supernatants on top of the hepatocyte pellets were collected after each spin and combined. The hepatocytes were resuspended in cold media (MEM Neaa, Gibco, Gaithersburg, MD) and placed on ice until further use. The collected supernatant was diluted 1:3 with cold S&M to remove the collagenase from the cells, and centrifuged at 1100 rpms (400 g) for 10 min to pellet the NPCs.
25 The NPC fraction was then suspended in 1X phosphate buffered saline (PBS) and stored at 4°C until oval cell isolation. The presence of oval cells in the NPC fraction has been previously determined (Yaswen, P., 1984, Cancer Research 44:324). The oval cells were then isolated from the NPC fraction using flow cytometric techniques as previously described (Petersen, B.E. et al., 1998, Hepatology 27:433). Briefly,
30 approximately 200×10^6 NPCs were incubated for 20 min at 4°C with fluorescein

isothiocyanate (FITC)-conjugated anti-rat Thy 1.1 (1 μ g/million cells) and then rinsed twice in 1X PBS + 1%FBS, 5 min each time. FITC isotype mouse IgG₁ was used as control. The cells were then kept on ice in the dark until sorted with a Becton Dickinson FACStar flow cytometer into Thy1.1⁺ (oval cells) and Thy1.1⁻ sub-
5 populations of cells. The purity of the different sorted cell populations were as follow: 95-97% pure for the Thy-1⁺ cells and 99% pure for the Thy-1⁻ cells (Petersen, B.E. et al., 1998, Hepatology 27:433).

6.1.7. *IN SITU* HYBRIDIZATION OF THE *SRY* REGION OF THE Y CHROMOSOME

10 Digoxigenin labeled DNA probes, prepared by random priming using the Genius System instructions (Boehringer Mannheim, Indianapolis, IN), were hybridized to paraffin or frozen liver sections as per manufacturer's instructions. Briefly, frozen sections were placed on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and were pre-hybridized in 10 mM Tris-HCl, 50%
15 formamide, 0.6 M NaCl, 1 mM EDTA, 1x Denhardt's, 0.5 mg/ml carrier RNA and 10% dextran sulfate for 1 hr at 37°C. The digoxigenin-labeled probe of the *sry* gene of the Y chromosome was applied to the sections and allowed to hybridize overnight at 37°C. Following post-hybridization washes, detection of the probe was accomplished by incubation with alkaline phosphatase-conjugated anti-digoxigenin
20 antibody (1:500) for 2 hr at RT. Alkaline phosphatase activity was visualized by incubation with NBT and BICP (Boehringer Mannheim, Indianapolis, IN) in the dark. The color development was monitored and the enzymatic reaction stopped by immersing the slides in 10 mM Tris, 1 mM EDTA.

6.1.8. IMMUNOHISTOCHEMISTRY AND CYTOCHEMISTRY

25 All tissue samples were divided into half. One was immediately placed in liquid nitrogen for later extraction of RNA or DNA. The other half was split, with one portion being fixed in 10% buffered formalin and processed for preparation of 4- μ m thick paraffin embedded sections, and the other portion placed in OTC freezing compound and cold 2-Methylbutane and processed for preparation of

6 μ m-thick frozen sections which were stored at -80°C until staining. Routine histological examinations were performed on both paraffin and frozen sections stained with hematoxylin and eosin (H&E). In addition, spleen and pancreas tissue were treated the same way and frozen sections and paraffin sections were DPPIV and
5 hematoxylin and eosin stained.

6.2. RESULTS

Female rats were lethally irradiated and rescued with a bone marrow transplant from a male animal. Nucleated blood cells of the transplanted animals were tested by PCR to establish that the BMTx was successful. Once the female rats were
10 determined to have been engrafted with male bone marrow, they were placed on the oval cell protocol as stated above. On Day 9 and Day 13 (post hepaic injury) rats were anesthetized and their livers were perfused in order to obtain single cell suspensions of non-parenchymal cells (NPC) (cells that are not hepatocytes) and parenchymal cells (primarily hepatocytes).

15 Flow cytometry was used to isolate the oval cells from the NPC fraction using anti-Thy-1-FITC. The Thy-1 positive, Thy-1 negative and hepatocyte populations were subjected to PCR analysis. Figure 1 shows that both the day 9 and day 13 Thy-1⁺ and Thy-1⁻ cell populations of NPCs were positive for the Y chromosome PCR product. The Thy-1⁻ fraction showed a strong signal, probably
20 due to the presence of donor hematopoietic cells such as Kupffer cells which are in the NPC population and Thy-1 negative, but positive for the Y chromosome. In the day 9 hepatocyte fraction there was no visible signal. At this time point in oval cell activation, the oval cells have not yet begun to differentiate into either transitional cells or hepatocytes. By day 13 there were cells in the hepatocyte fraction expressing
25 the Y chromosome PCR (549 bp) product. At this time the oval cells are beginning to differentiate into hepatocytes (Grishman, J.W., 1997, in Stem Cells, C.S. Potten, Ed. (Academic Press, San Diego, CA p233-282). If all oval cells that differentiate into hepatocytes were derived from the liver, then one would expect that none of the hepatocytes tested would be positive for the Y chromosome. The finding that some
30 hepatocytes were Y chromosome positive indicates that they were derived from the

bone marrow donor cells. The combined data indicates that at day 9 the oval cells (Thy-1⁺) in the recipient female were derived from the donor male and that they continued to differentiate into mature hepatocytes by day 13.

To confirm the PCR results seen in Figure 1, one of the smaller lobes from every liver that was perfused was ligated, removed and split in half. One portion was fixed in 10% buffered formalin and processed into 4- μ m thick paraffin embedded sections; the other portion was placed in OTC freezing compound and processed into 6 μ m-thick frozen sections. *In situ* hybridization for the Y chromosome *sry* gene was performed on both types of fixed tissue. Hepatocytes carrying a positive reaction product (blue staining) in their nuclei were readily seen in untreated control male rats (Figures 2A). In agreement with the results obtained by PCR analysis of the isolated hepatocyte fraction, cells with positive blue staining (Y chromosome positive) were detected in females subjected to BMTx and the 2-AAF/CCl₄ protocol at day 13 (Figure 2B). Figure 2C shows no reaction product in the liver of an untreated control female.

In the second set of bone marrow transplantation experiments, bone marrow cells from DPPIV⁺ F-344 male rats were injected into lethally irradiated DPPIV⁻ F-344 females. This constituted a system in which the presence of cells originating from donor cells in the recipient liver could be easily detected, by revealing cytochemically the activity of the enzyme DPPIV (Figure 3). As previously reported with studies of this enzyme, a diffuse red to brownish-red staining of the bile canalicular site between hepatocytes was observed. This type of staining was seen in the DPPIV⁺ F-344 male rats (Figure 3A). The control untreated DPPIV⁻ females showed no staining (Figure 3B). To determine whether DPPIV would be expressed in transplanted animals, staining was performed on liver sections prepared from transplanted female rats treated with 2-AAF/CCl₄. As seen in Figure 3C-3F, DPPIV expression was observed in several bile canalicular sites between hepatocytes from four different transplanted animals. DPPIV expression was also observed on a few oval cells/transitional (small hepatocyte) cells in the liver from these rats (Figure 3D and 3E).

Four animals showing the strongest evidence of the donor marrow engraftment, based upon examination of the recipient rat's spleens, were chosen

(Figure 3C-3F) for an estimation of the number of hepatocytes originating from the donor bone marrow. From these four animals, five different lobes were used to cut approximately 10 sections per lobe (50 sections total). All of the sections were stained for DPPIV and examined for DPPIV expression. Roughly, 25 random fields (200x) per section were examined, and the numbers indicate the total number of hepatocytes positive for DPPIV staining per section.

Table 1 represents the number of DPPIV positive hepatocytes observed in the transplanted DPPIV⁻ rats. By dividing the total positive cells by the total hepatocytes observed, approximately 0.16% of the total number of hepatocytes were positive for DPPIV expression. The rat liver has approximately 700×10^6 hepatocytes, indicating that, at day 13, approximately 1.0×10^6 hepatocytes originated from transplanted bone marrow cells using the oval cell protocol.

TABLE 1. Percent of DPPIV positive hepatocytes in BMTx DPPIV deficient females					
Animal Number	LOBES # of Different	SECTIONS # of Different non-serial	FIELDS Total # of Random Fields	DPPIV+ Total # of positive Hepatocytes	PERCENT TOTAL
6028	5	10	250	55	0.144
6034	4	10	200	18	0.059
6036	5	10	250	78	0.204
6037	5	10	250	93	0.243

The mean value of 0.163% represents that approximately 1×10^6 hepatocytes would be positive for DPPIV expression within the transplanted female rats.

In order to confirm that extra-hepatic cells can repopulate the liver, whole liver transplantation was used as a final approach. In this series of experiments, Lewis rats that express the MHC class II L21-6 isozyme were used as recipients of liver from Brown-Norway rats which do not express L21-6 (Yagihushi, A. et al., 1995, Transplantation Proceedings 27:1519). An anti L21-6 monoclonal antibody was used to differentiate donor from recipient cells. In this model, oval cells that

originated from an extra-hepatic source would be L21-6 positive, while oval cells originating *in situ* would be negative. The results obtained in these experiments are illustrated in Figures 4 and 5. Figure 4A and 4B shows stained sections of two Brown-Norway rat livers transplanted into Lewis rats prior to being placed on the 2-AAF/CCl₄ protocol. A widespread staining of L21-6 was present in the transplanted Brown-Norway livers, presumably due to influx of cells of the Lewis host immune system reacting to the allogenic Brown-Norway liver. Most notable, though, was the presence of ductal structures containing L21-6 positive cells. These structures represent a pattern often seen in the organization and differentiation of actively proliferating oval cells (Thorgeirsson, S.S. et al., 1993, Proc. Soc. Exp Biol Med 204:253; N. Fausto, in *The Liver: Biology and Pathobiology* I.M. Arias et al. Eds (Raven Press, New York 1994) p. 1501-1518 (third edition)). The presence therein of positive cells supports the concept that these oval cells were derived from an extra-hepatic source. The structures also contained cells which were clearly L21-6 negative, suggesting that some oval cells were derived *in situ* from the donor liver (Figure 4B).

To better characterize the positive cells seen in the ductal structures and distinguish them from inflammatory cells invading the Brown-Norway transplanted livers, double immunofluorescence staining was performed using an antibody against OC.2, a specific oval cell/ductal cell marker (Faris, R.A. et al., 1956 Cancer Research 16:142), in conjunction with the L21-6 antibody. As can be seen in Figure 5, individual cells expressing both OC.2 and L21-6 are evident as a yellow stain (combination of red and green). The cells, therefore, were identified as oval cells or derivatives thereof, and not immunocytes or inflammatory cells.

In addition, when tissues other than the liver were analyzed, such as the pancreas and spleen, and cell staining was observed, indicating that transplanted bone marrow cells were capable of infiltrating and incorporating into these tissues. As indicated in Figure 17A-F, when experimental DPPIV deficient rats were transplanted with DPPIV positive bone marrow, a large number of cells were positive for DPPIV (Figure 17 E-F).

7. EXAMPLE: PURIFICATION OF HEPATIC OVAL
CELLS USING ANTIBODIES THAT RECOGNIZE THE
THY1.1 CELL SURFACE ANTIGEN

The following subsection discloses experimental data indicating that
5 hepatic oval cells express the hematopoietic stem cell marker Thy1.1. The data
provides a novel cell marker for identification of oval cells. Using Thy1.1 antibody, a
highly enriched population of oval cells was obtained.

7.1 MATERIALS AND METHODS

CCl₄, 99% pure high-performance liquid chromatography grade, and 2-
10 AAF were purchased from Aldrich Chemical Co. (St. Louis, MO). 2-AAF crystals
were incorporated into time-released pellets (70 mg/pellet over a 28-day release, 2.5
mg/d) supplied by Innovative Research Inc. (Sarasota, FL). Male Fisher 344 rats
(150-170 g) were obtained from Frederick's Laboratories (Frederick, MD).
Microscope Superfrost Plus slides, buffered Formalin-Fresh, and dextran sulfate were
15 obtained from Fisher Scientific (Pittsburg, PA). Hematoxylin was purchased from
Anatech, Ltd. (Battle Creek, MI). Anti- α -fetoprotein (AFP) antibody was purchased
from Nordic Immunology (Tilburgh, the Netherlands). OV-6 and BD-1 antibodies
were gifts from Dr. Doug Hixson (Brown University, Providence, RI). OC.2 antibody
was a gift from Dr. Ron Faris (Brown University, Providence, RI). Rat anti-GGT was
20 a gift from Dr. Benito Lombardi (University of Pittsburgh, Pittsburgh, PA). Thy1.1
was purchased from PharMingen Inc. (San Diego, CA). Proliferating cell nuclear
antigen (PCNA) was purchased from Signet Laboratory Inc. (Dedham, MA). 5-
Bromodeoxy- uridine (BrdU) was obtained from Boehringer Mannheim (Indianapolis,
IN). Desmin was obtained from Dako Corp. (Carpinteria, CA). Eosin, propidium
25 iodide (PI), and all other chemicals used were obtained from Sigma Chemical
Company (St. Louis, MO).

7.1.2 COMPOUND DELIVERY

An LD₅₀ dose of CCl₄ as determined by Lewis was used (SAX Dangerous Properties of industrial Material, In: Lewis, R.J., Sr. Edition 8, 1993 update and vol 3. New York: Van Nostrand Reinhold, 1992 52:1149). A single dose of 1.9 mL/kg (1,500 mg/kg) of body weight, in a 1:1 vol/vol dilution in corn oil, was administered by intraperitoneal injection. Two hours before they were sacrificed, the animals received an intra peritoneal injection of BrdU (50 mg/kg body weight) to identify cells in S-phase of the cell cycle, as described by Lindrose et al (1991, Hepatology 13:743-750).

7.1.3 OVAL CELL COMPARTMENT PROLIFERATION/ACTIVATION

2-AAF pellets were inserted 7 days before hepatic injury following a protocol similar to Novikoff et al. (1996, Am J Pathol 148:1473-1491) and Hixson et al. (1990, Pathobiology 58:65-73) The time points for this study were counted from when the hepatic injury (CCl₄, Phx) was induced. The dose and delivery for CCl₄ was discussed earlier in the compound delivery section and performed in the same manner. For the Phx procedure, rats were hepatectomized under general anaesthesia according to the methods described by Higgins and Anderson (1931, Arch. Pathol. 12:186-202). The tissue obtained was processed in the same manner described in the immunohistochemistry methods.

7.1.4 ANIMAL EUTHANIZATION

All procedures involving animals were conducted according to institutionally approved protocols. Rats were anesthetized by injection with sodium pentobarbital (0.1 mL/100 g body weight) before being sacrificed.

7.1.5 IMMUNOHISTOCHEMISTRY

A basic immunohistochemical protocol previously described by Wolf et al. (1991, Hepatology 12:186-202) was used with slight modification to conform to each particular antibody. Liver tissue was divided and fixed in either 10% buffered formalin or placed in OTC compound, frozen in cold 2-methylbutane (Fisher

Scientific), and stored at -80°C. All staining procedures for light microscopy were performed on 4-µm thick, paraffin-embedded sections or 6-µm thick, frozen sections. Routine histological examination were made for all liver tissue samples on sections (paraffin and frozen) stained with hematoxylin-eosin. Single cell suspensions were collected on glass slides by cytocentrifugation and air-dried. Cytocentrifugation was performed using a Cytospin 3 Cytocentrifuge (Shandon Inc. Pittsburgh, PA) for 6 minutes at 600 rpm. Immunohistochemistry on cytopsin preparations (100,000 cells/slide) was performed using the techniques described above. The cytopsin preps were then analyzed by confocal microscopy (Multiprobe 2001 Inverted Confocal Laser Scanning Microscope, CLSM, Molecular Dynamics, Sunnyvale, CA). BrdU staining was performed on 4-µm thick, paraffin-embedded tissue as described by Lindroos et al. (1991, Hepatology 12:186-202). For each, antibody-negative controls were performed by either blocking with the appropriate nonimmune serum or by omitting the primary antibody from the protocol.

15

7.1.6. FLOW CYTOMETRY

Hepatocyte and nonparenchymal cell isolation was performed by a two-step collagenase digestion according to the protocol established by Seglen (1976, Methods in Cell Biol 13:29-83). Oval cell isolation was performed using flow cytometry. Briefly, the nonparenchymal cell (NPC) fraction has been determined to contain the hepatic oval cell population as described by Yaswen et al. (1984, Cancer Res 44:324-331). Immunohistochemistry was performed on the parenchymal and NPC fractions to ensure that the cells of interest were in the NPC fraction. The NPC fraction was found to contain the highest percentage of oval cells. A portion (approximately 60 to 80 x 10⁶ of the total 200 x 10⁶ cells) of the NPC fraction was further purified using flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated anti-rat Thy1.1 (1 mg/million cells) was used to label the target cells. The cell fraction was incubated with the antibody for 20 minutes at 4°C, rinsed twice in 1x phosphate-buffered saline + 1% fetal bovine serum for 5 minutes each, and stored in the dark on ice until sorting. A FITC mouse G₁ IgG₁ was used as an isotype control. Cells were sorted using a Becton Dickinson FACStar flow cytometer into two

populations: Thy1.1-positive (Thy1.1⁺) and Thy1.1-negative (Thy1.1⁻). Those animals whose livers were perfused had one of the smaller lobes surgically removed before the cell isolation procedure. This tissue was used as an internal control for light microscopy, as well as for *in situ* hybridization. The excised liver tissue was divided in half and fixed in either 10% buffered formalin or placed in OTC compound, frozen in cold-2-methylbutane (Fisher Scientific), and stored at -80°C.

Cell Cycle Analysis. One x 10⁶ Thy1.1⁺ cells were fixed in ice-cold 70% ethanol at 4°C overnight. Following centrifugation (5 minutes at 3,000 rpm), the ethanol was removed and 1 mL of propidium iodide staining solution consisting of 50 mg/mL PI, 100 U/mL RNase A in Ca- and Mg-free phosphate-buffered saline + glucose was added and incubated for 30 minutes at room temperature. Flow cytometric cell cycle analysis was then performed.

7.2 RESULTS

7.2.1 TIME LINE OF EVENTS FOR ACTIVATION OF OVAL CELL PROLIFERATION

To activate oval cell proliferation in the liver, certain events must occur. Figure 6 is an outline that represents the events and times involved in the process of the activation of oval cell proliferation. In control animals, a placebo was inserted in place of 2-AAF. Oval cells can be seen as early as day 5 posthepatic injury and can still be detected as late as day 42 postinjury. Figure 7A-B shows the histological changes in liver sections from rats exposed to 2-AAF for 7 days, followed by CCl₄ exposure and sacrificed 11 days posthepatic injury. With the 2-AAF/CCl₄ protocol, massive oval cell proliferation was seen after day 9. The sections were also stained with antibodies for specific oval cell markers (e.g., CK-19 and GGT), and the oval cells generated from the 2-AAF/CCl₄ protocol were positive for these markers. The major peak of BrdU incorporation for oval cells occurred at day 9 posthepatic injury, with a drastic drop-off in BrdU incorporation on subsequent days. These results can be seen in Figure 8A-D, which shows livers sections from rats sacrificed on day 9 (Figure 8A), day 11 (Figure 8B), and day 13 (Figure 8C) post-CCl₄ exposure. The same type of proliferation pattern was also seen in the 2-AAF/Phx model.

7.2.2. IMMUNOHISTOCHEMISTRY FOR THY-1 ON ACTIVATED HEPATIC OVAL CELLS

To determine whether Thy-1 and OC.2 antigens are expressed only by
oval cells, normal rat liver was first examined. Figure 4 represents frozen sections
5 obtained from normal rat liver. These sections were stained with Thy-1.1 antibody
(Figure 9A) or OC.2 antibody (Figure 9B). There appears to be no portion of the liver
expressing the Thy-1 antigen. Figure 9B represents OC.2 staining. The ductular cells
appear to be positive with little to no staining elsewhere in the liver. Our results are in
agreement with Faris et al. (1991, Cancer Res 51:1308-1317), who previously
10 reported this pattern of staining for OC.2 in normal liver.

To test for Thy-1 expression on oval cells, frozen sections of livers
with proliferating oval cells were used. In addition to Thy-1 expression, CK-19 and
OC.2 expression was also examined. Figure 10 represents frozen liver sections
obtained from rats on the 2-AAF/CCl₄ protocol at day 21 after liver injury. These
15 sections were stained for CK-19 (Figure 10A and 10D), OC.2 (Figure 10B and 10E),
and Thy-1 (Figure 10C and 10F). Staining by all three antibodies on serial sections
showed similar patterns, with all staining located in the periportal region and
spreading outward; the pericentral region is devoid of staining. At higher
magnification (Figure 10 D-F), the reactivity of the antibodies was primarily to the
20 oval cells (arrows), or what has been termed in the art as transitional cells. Thy-1
antibody stained the ductular epithelium, which showed negative staining. This is
also evident in Figure 11.

The possibility that cells staining positive for Thy-1 may be
inflammatory cells in response to the CCl₄ exposure was ruled out. In the 2-AAF/PHx
25 model, however, there is presumably no influx of inflammatory cells. Figure 11
shows sections from livers with oval cells, in which the hepatic injury was a PHx of
two thirds (day 13 2-AAF/PHx). Again, as shown in Figure 10, the pattern of staining
in the periportal region of the lobule and expanding outward was similar to the
staining pattern seen in livers from a 2-AAF/PHx model (Figure 11A-C). At a higher
30 magnification (Figure 11D-F), the staining appeared localized to the oval cell
population. These cells were positive for all three markers (CK-19 (Figure 11A and

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11D); OC.2 (Figure 11B and 11E); Thy-1 (Figure 11C-11F)). Clearly, with both models of hepatic injury (2-AAF/CCl₄, 2-AAF/PHx), the pattern of staining for CK-19, OC.2, and Thy-1 was the same within the same population of cells. It should also be noted that frozen sections from both 2-AAF/CCl₄ and 2-AAF/PHx were double-labeled with Thy-1 FITC and OC.2-Texas Red, and the same staining pattern was seen.

7.2.3. FACS ANALYSIS AND SORTING

FOR HOC USING THE THY-1 MARKER

As shown above, using immunohistochemistry, oval cells express Thy1.1 antigen. The possibility of obtaining a distinct Thy-1⁺ population by cell sorting was next tested. Figure 12 represents FACS analysis of Thy-1⁺ and Thy-1⁻ sorted oval cells obtained from day 12 liver on the 2-AAF/CCl₄ protocol. Both the forward-/side-scatter plots and histograms are shown. Figure 12A (right gate) is that of Thy-1⁺ cells, and Figure 12B (left gate) is Thy-1⁻ cells. The Thy1.1⁺ population was sorted to 97% purity and the Thy1.1⁻ cells to a 99% purity. In each experiment, only a portion of the NPC fraction was sorted (60 to 80 x 10⁶ cells) and typical yields of Thy-1⁺ cells obtained were 15 to 20 x 10⁶ cells. These two populations of sorted cells were 95% viable by Trypan blue exclusion. The Thy1.1⁺ sorted cells were further examined to determine what stage of the cell cycle they were in. Oval cells were stained with propidium iodide and analyzed. Cell cycle analysis in Figure 12C shows a majority (>90%) of the Thy1.1⁺ cells were in the G0/G1 stage of the cell cycle. Figure 12D shows that ~90% of those cells in the G0/G1 stage of the cell cycle were Thy-1⁺.

Immunohistochemical staining for BrdU and PCNA (Figure 13A and 13B, respectively) also revealed that the oval cells were not in the cell cycle at the stage of the model used for this study. Only a few cells were positively stained for either marker, representing the second peak in the propidium iodide analysis (Figure 13C and 13D). In a separate study, it was shown that there was only one peak (day 9) of BrdU incorporation for oval cells (2-AAF/CCl₄ protocol), and the days following the peak showed very little incorporation. Others have shown that oval cell

proliferation lasts much longer, and perhaps the differences can be explained by the differences in the protocols. The data shown here were from experiments using the 2-AAF/ CCl_4 protocol.

7.2.4. IMMUNOHISTOCHEMISTRY ON THY-1⁺ SORTED HOC

5 Having obtained Thy-1⁺ and Thy-1⁻ populations, the sorted
populations were further characterized to determine if the traditional oval cell markers
were expressed. Thy-1⁺ and Thy-1⁻ cells were cytocentrifugated onto slides. Figures
14 and 15 represent Thy-1⁺ and Thy-1⁻ cells, respectively. Figure 14A is a
hematoxylin-eosin stain, and Figure 14B is a representative of a negative control in
10 which the primary antibody was omitted. For all antibodies, the appropriate negative
controls were performed, either by omitting the primary antibody or by blocking with
an appropriate nonimmune serum. Figure 14C-14F is of sorted Thy-1⁺ cells stained
with α -fetoprotein (AFP), CK-19, GGT, and OV6, respectively. To show that the
cells of interest (Thy-1⁺ cells) are not Ito cells, staining for desmin, an Ito cell-specific
15 marker, was performed. These data are shown in Fig. 14G. The Thy-1⁺ cells were
negative for desmin, which indicated that these cells were Ito cells. Also, desmin
staining was performed on liver sections obtained from both 2-AAF/ CCl_4 - and 2-
AAF/PHx-treated animals at various time points, and the oval cells were also
negative. The results shown in Fig. 14H are of double-stained oval cells showing
20 Thy-1-FITC (green) and AFP-Texas Red (red). In regions of the cell in which the
antibody binding is in close proximity to each other, the two fluorochrome
wavelengths mix, and the resulting fluorescence is yellow. The presence of dual
markers (yellow) was evident in most cells shown. In addition, both Thy-1 (green) and
AFP (red) were seen as distinct colors in separate cellular domains on the same cell.
25 To show that the Thy-1⁺ staining was specific for oval cells, Thy-1⁻
cells were also subjected to the same immunostaining described above. Figure 15
represents these Thy-1⁻ cells; Figure 15A is a representative hematoxylin-eosin stain
of Thy-1⁻ cells. Figure 15B and 15D are cells stained for AFP and GGT, respectively.
Their corresponding negative controls are shown in Fig. 15C and 15E, respectively.
30 In both cases, the Thy-1⁻ populations were devoid of any staining for these two

traditional oval cell markers. The specificity for Thy-1 binding appeared to be only specific to oval cells, as seen in Figures 14 and 15.

5 The present invention is not to be limited in scope by the specific embodiments described herein which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such
10 modifications are intended to fall within the scope of the claims. Various publications are cited herein, the contents of which are hereby incorporated, by reference, in their entireties.

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CLAIMS

1. A method for stimulating liver regeneration in a subject having a liver disorder comprising administering of bone marrow cells to said subject in an amount sufficient to result in the production of hepatocytes, bile ductal cells and/or oval cells.
2. The method of Claim 1 wherein the bone marrow cells are injected.
3. The method of Claim 1 wherein the bone marrow cells are transplanted into the liver.
4. The method of Claim 1 wherein the bone marrow cells are genetically engineered to express a functionally active protein.
5. The method of Claim 1 wherein the bone marrow cells are on a support matrix.
6. A method for stimulating liver regeneration in a subject having a liver disorder comprising the administration of enriched oval cells to a subject in an amount sufficient to result in the production of hepatocytes, bile ductal cells and/or oval cells.
7. The method of Claim 6 wherein the oval cells are injected.
8. The method of Claim 6 wherein the oval cells are transplanted into the liver.
9. The method of Claim 6 wherein the oval cells are genetically engineered to express a functionally active protein.

10. The method of Claim 6 wherein the oval cells are on a support matrix.
11. The method of Claim 1 or 6 wherein the dose of cells is between 10^5 - 10^6 .
- 5 12. The method of Claim 1 or 6 wherein the dose of cells is between 10^6 - 10^8 .
13. The method of Claim 1 wherein the bone marrow cells are contacted with a growth factor prior to administration.
- 10 14. The method of Claim 6 wherein the oval cells are contacted with a growth factor prior to administration.
- 15 15. A method for stimulating pancreatic regeneration in a subject having a pancreatic disorder comprising administering of bone marrow cells to said subject in an amount sufficient to result in the production of pancreatic cells.
16. The method of Claim 15 wherein the bone marrow cells are injected.
17. The method of Claim 15 wherein the bone marrow cells are transplanted into the pancreas.
- 20 18. The method of Claim 15 wherein the bone marrow cells are genetically engineered to express a functionally active protein.
19. The method of Claim 15 wherein the bone marrow cells are on a support matrix.

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20. A method for enriching for oval cells comprising:
- (a) disaggregating liver tissue to form a single cell suspension of hepatic cells;
 - (b) purification from the single cell suspension of Thy1.1 positive hepatic cells from Thy1.1 negative hepatic cells;
- 5 wherein the Thy1.1 positive cells comprise the oval cells.
21. The method of Claim 20 wherein Thy1.1 positive hepatic cells are purified from Thy1.1 negative cells using a Thy-1 specific antibody.
22. A composition comprising an enriched population of oval cells in a physiologically acceptable carrier.
- 10 23. A composition of matter comprising an enriched population of oval cells attached to a matrix.
24. The composition of matter of Claim 23 further comprising a growth factor associated with the matrix.

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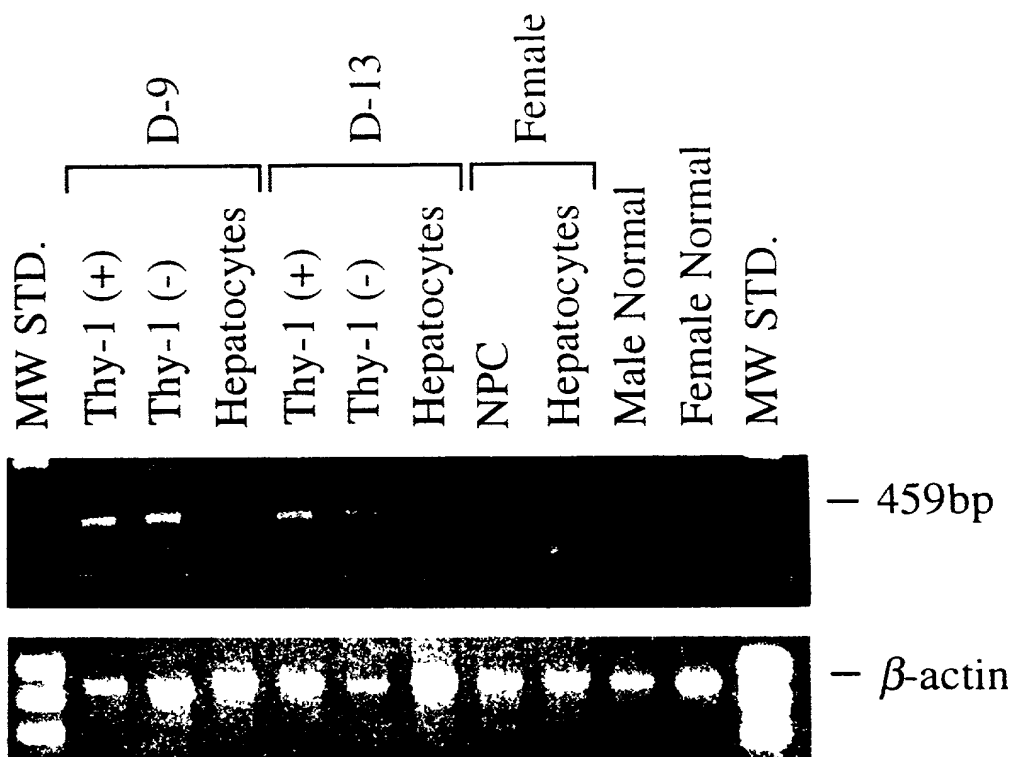


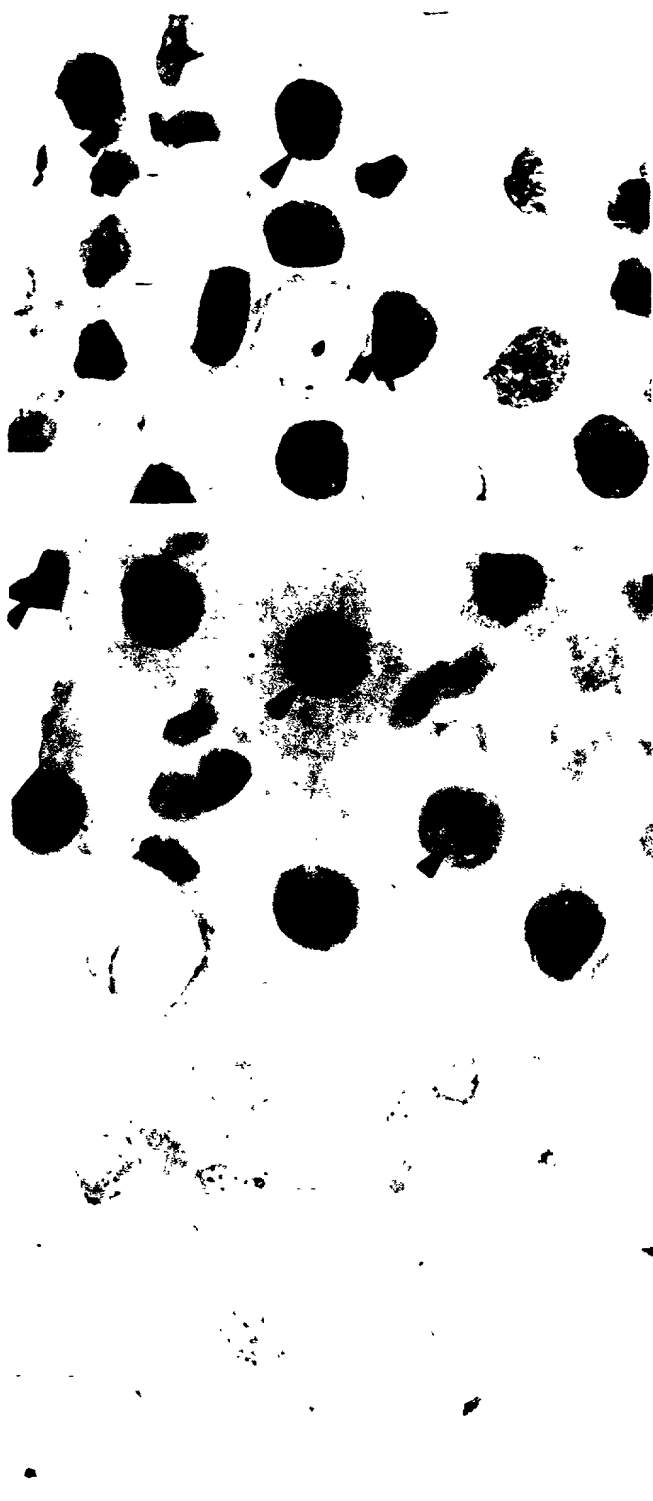
FIG.1

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FIG.2A

FIG.2B

FIG.2C



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FIG.3B



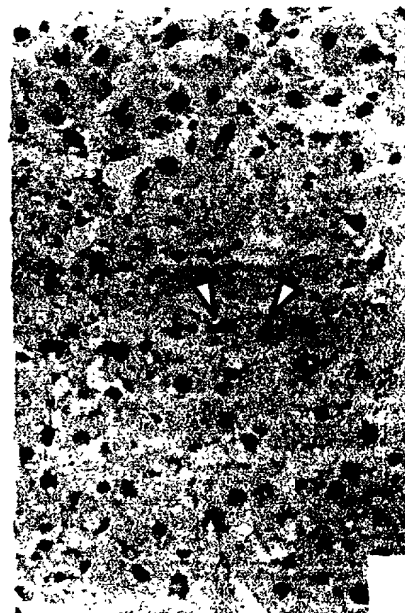
FIG.3D



FIG.3A



FIG.3C



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FIG.3F

FIG.3E



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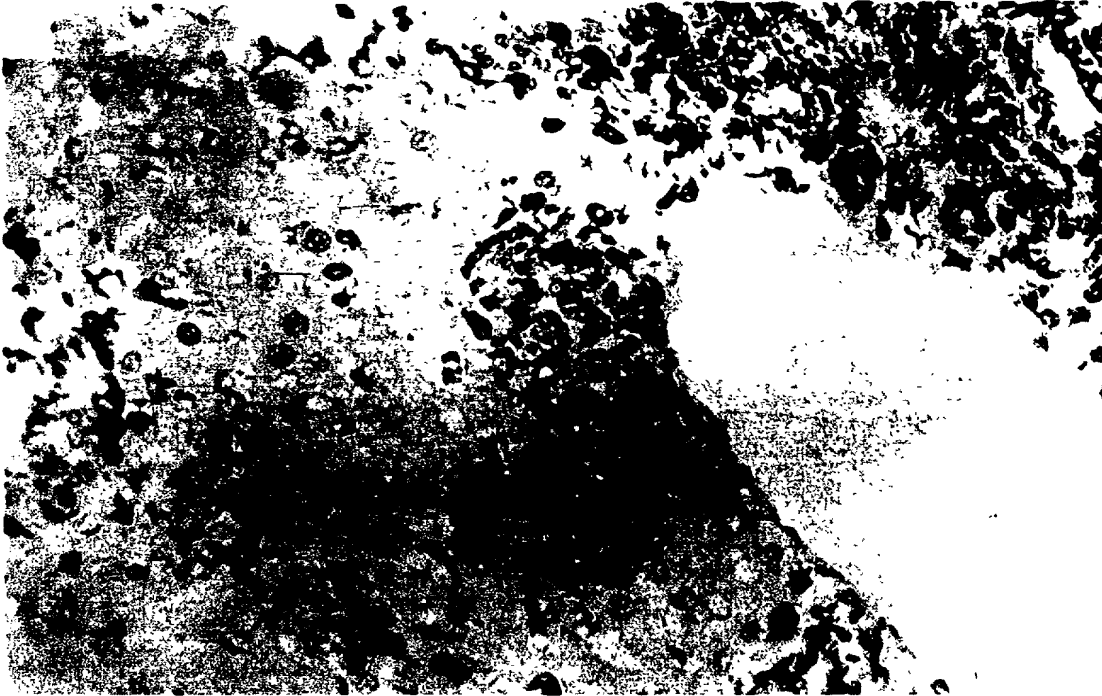


FIG.4A



FIG.4B

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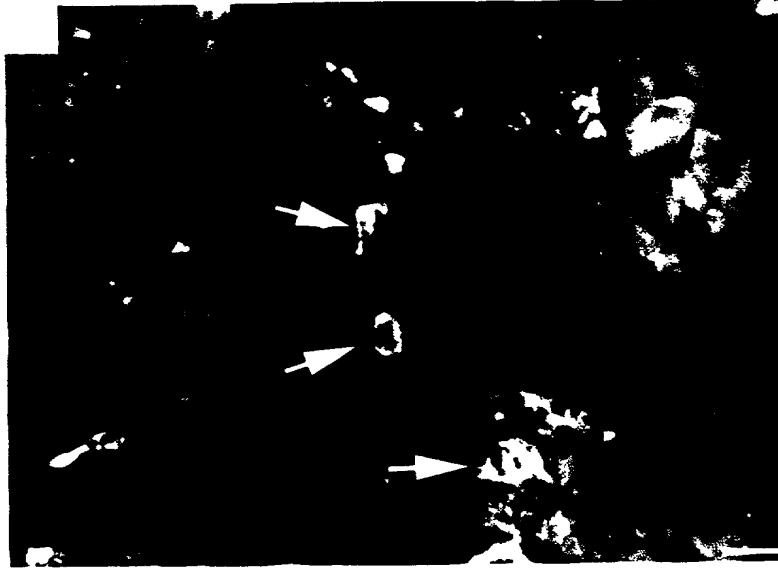


FIG. 5A

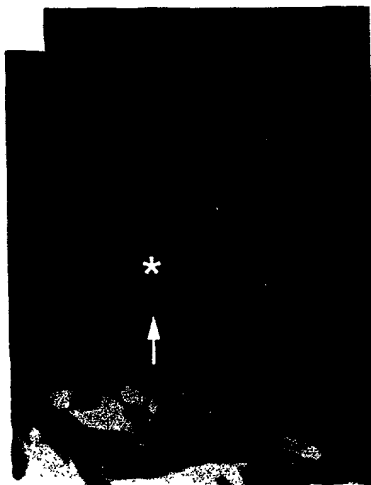


FIG. 5B

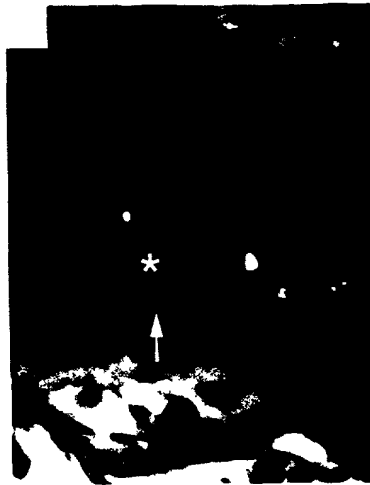


FIG. 5C

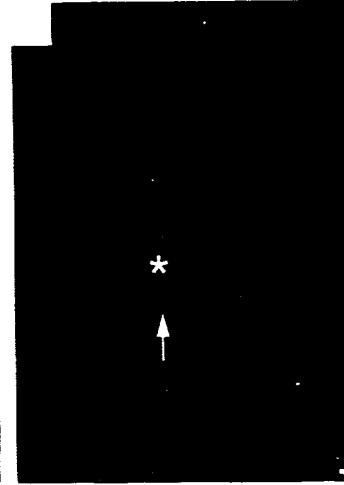


FIG. 5D

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2-AAF PELLET
(INSERTED SUBCUTANEOUSLY)

5-7 DAYS

LIVER INJURY
i.e. CCl_4 , PhX

5-21 DAYS

OVAL CELL
ACTIVATION

?

BILE DUCT CELLS

HEPATOCYTES

FIG.6

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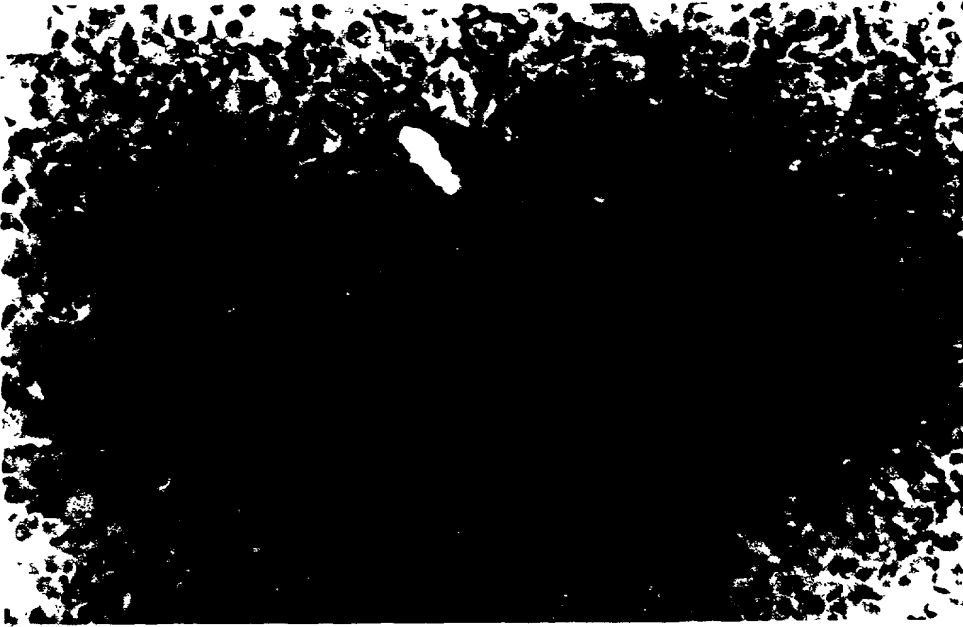


FIG.7A

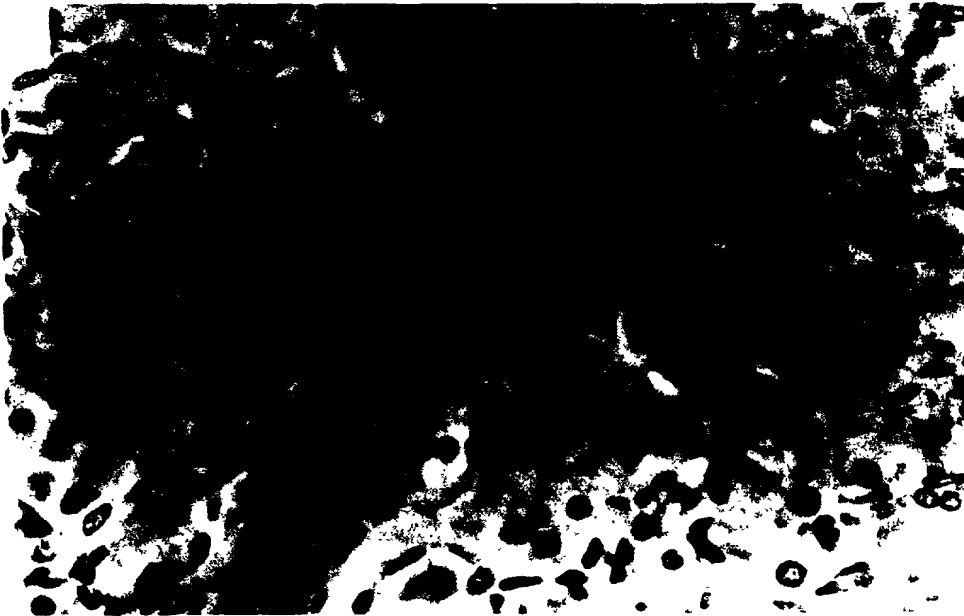


FIG.7B

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FIG. 8B

FIG. 8A

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FIG.8D

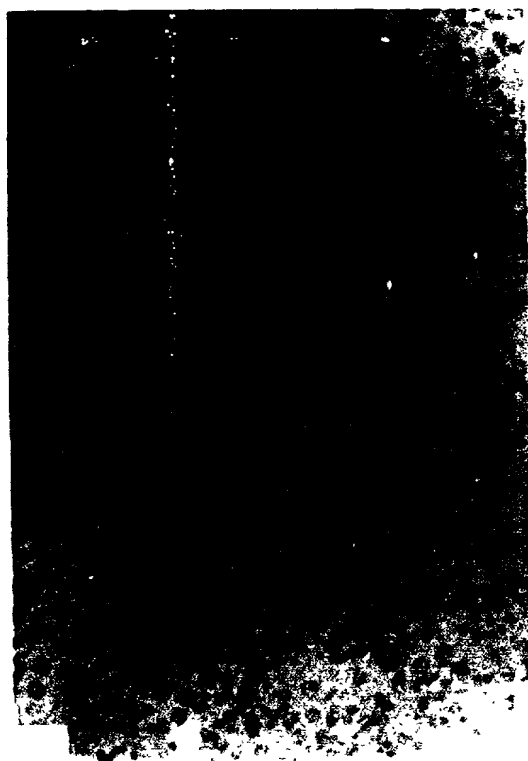


FIG.8C

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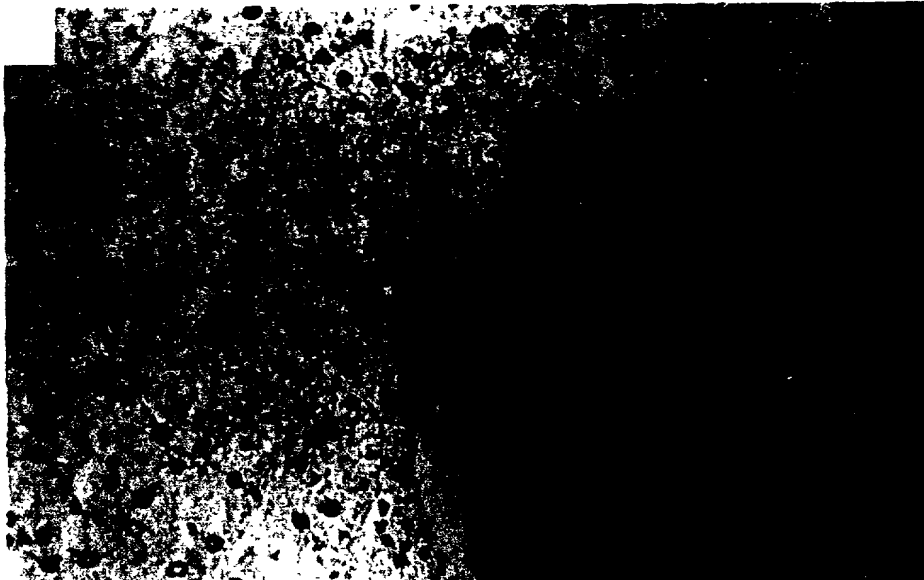


FIG.9A



FIG.9B

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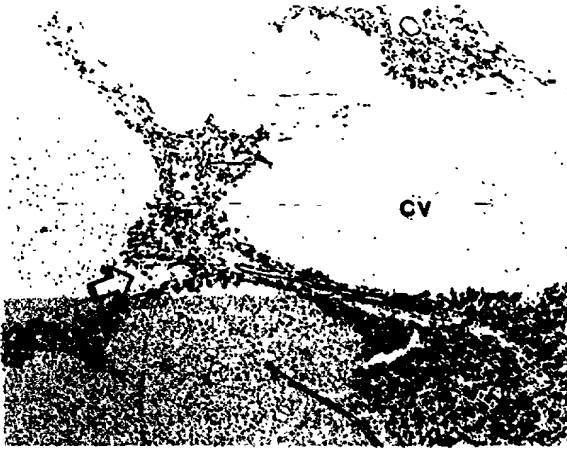


FIG.10A

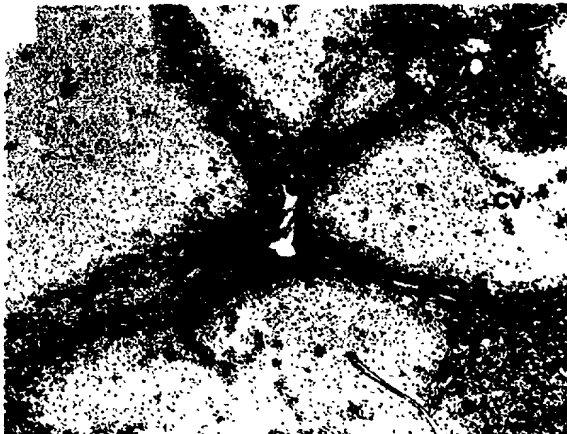


FIG.10B

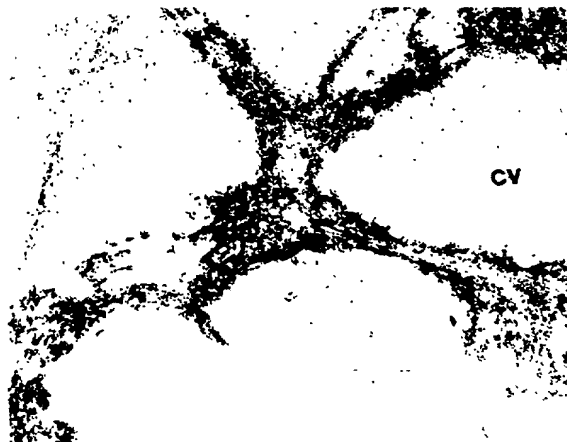


FIG.10C

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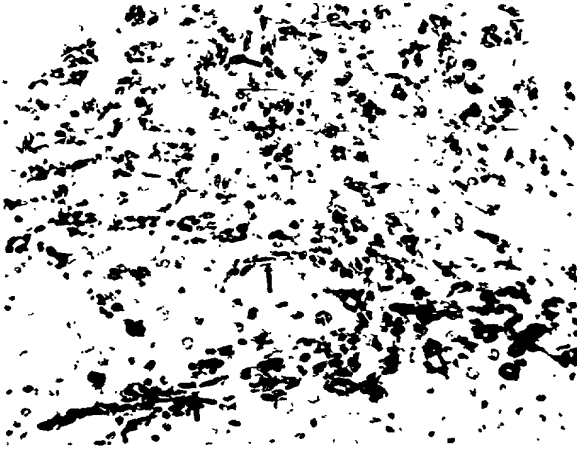


FIG.10D

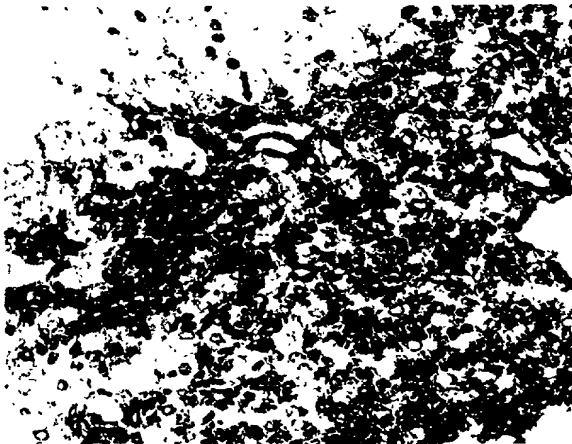


FIG.10E

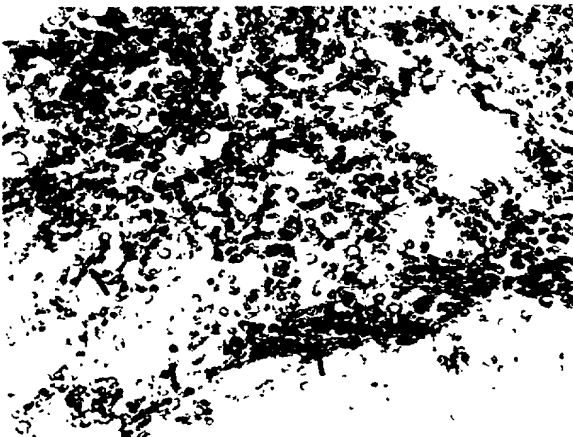


FIG.10F

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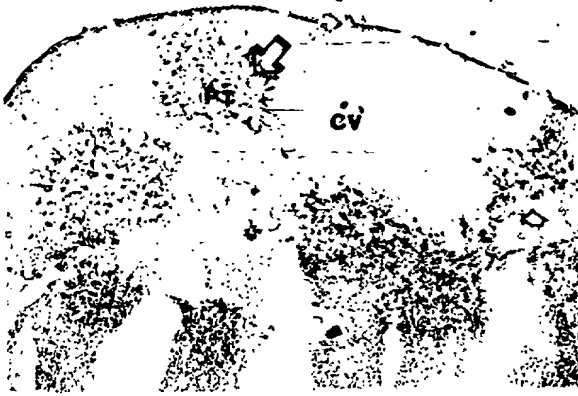


FIG.11A



FIG.11B



FIG.11C

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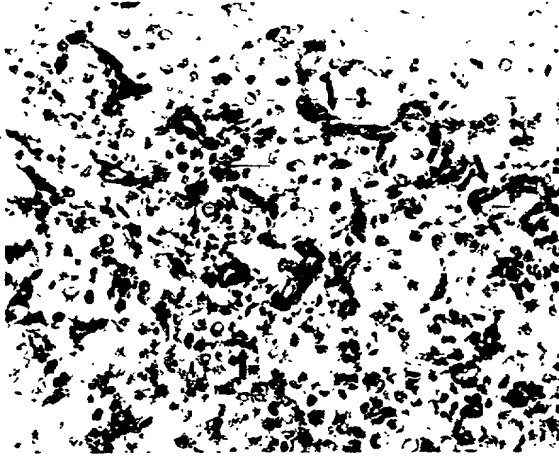


FIG.11D



FIG.11E

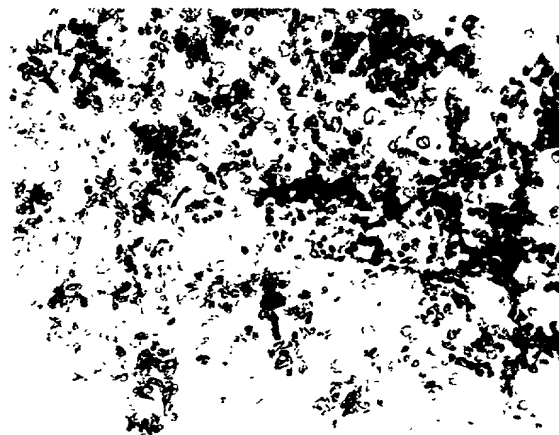


FIG.11F

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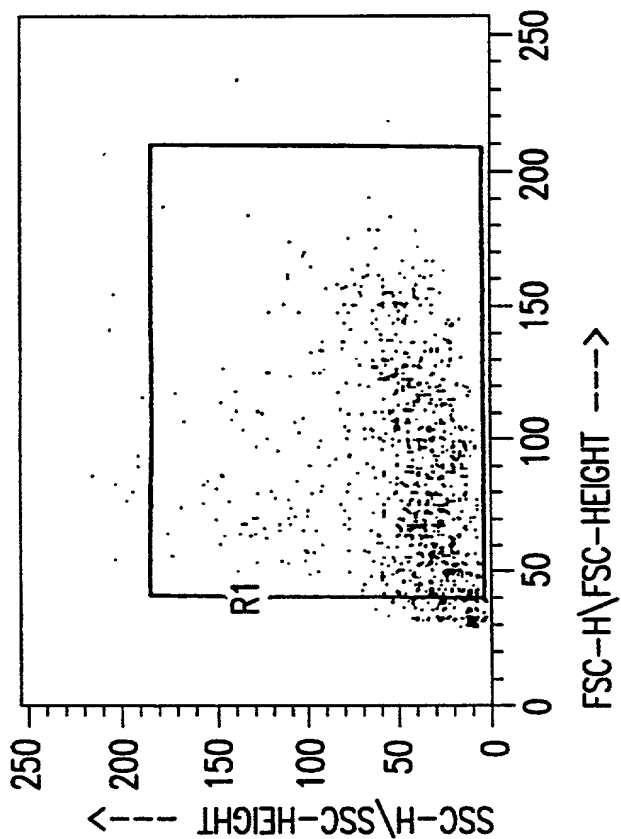


FIG.12A

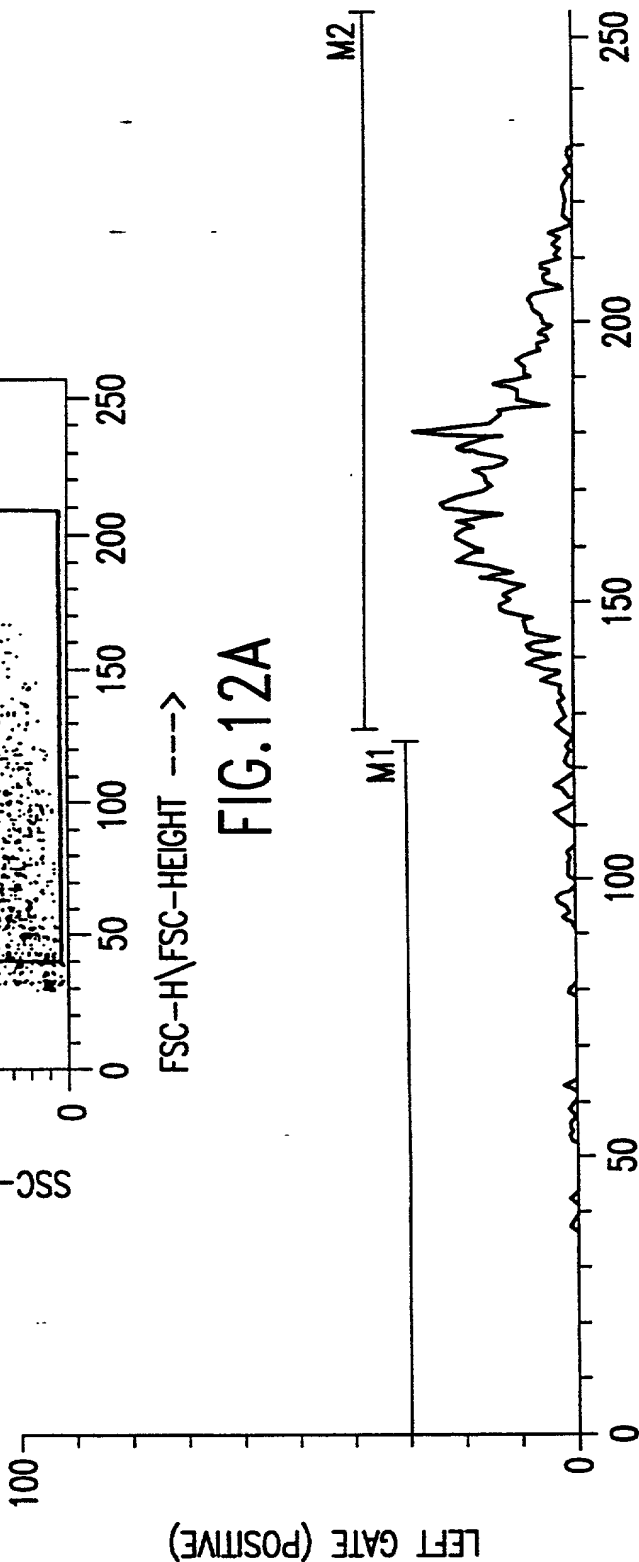


FIG.12A-1

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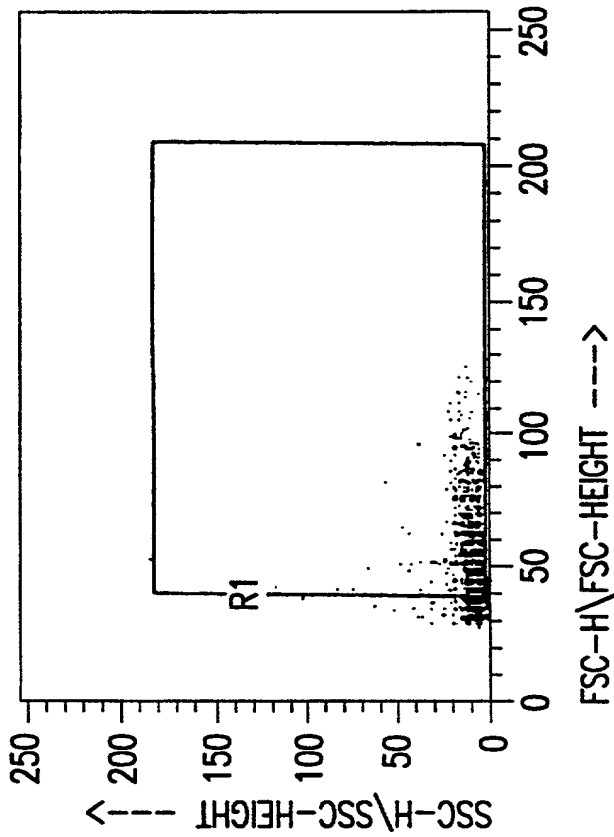


FIG.12B

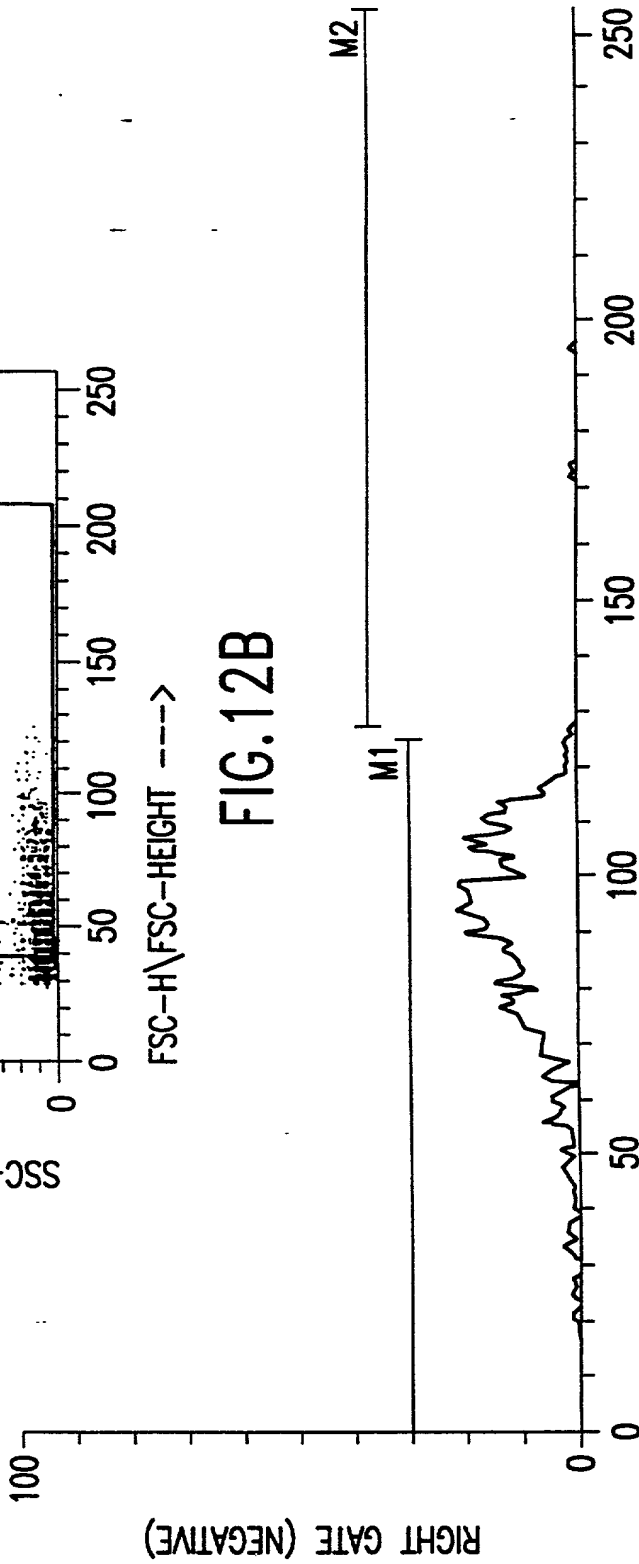


FIG.12B-1

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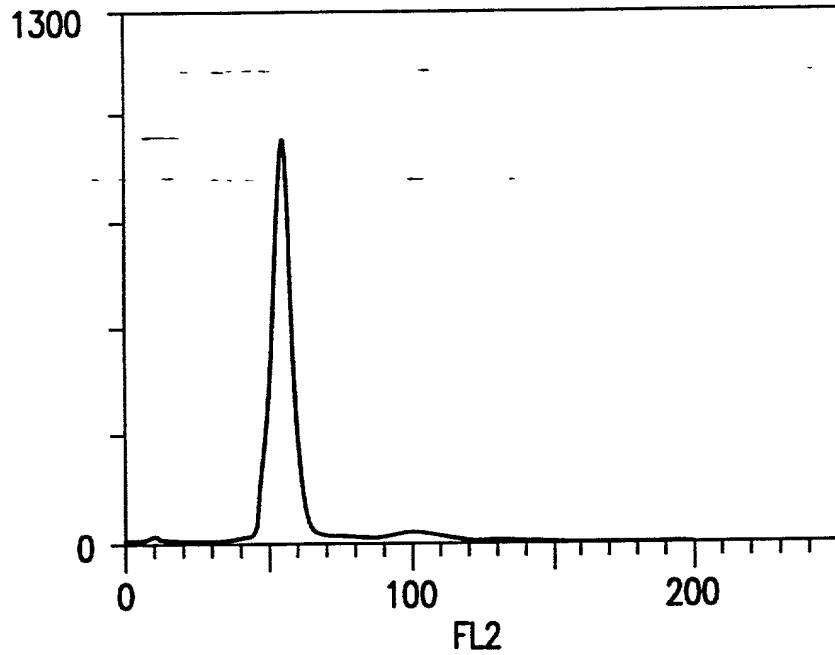


FIG.12C

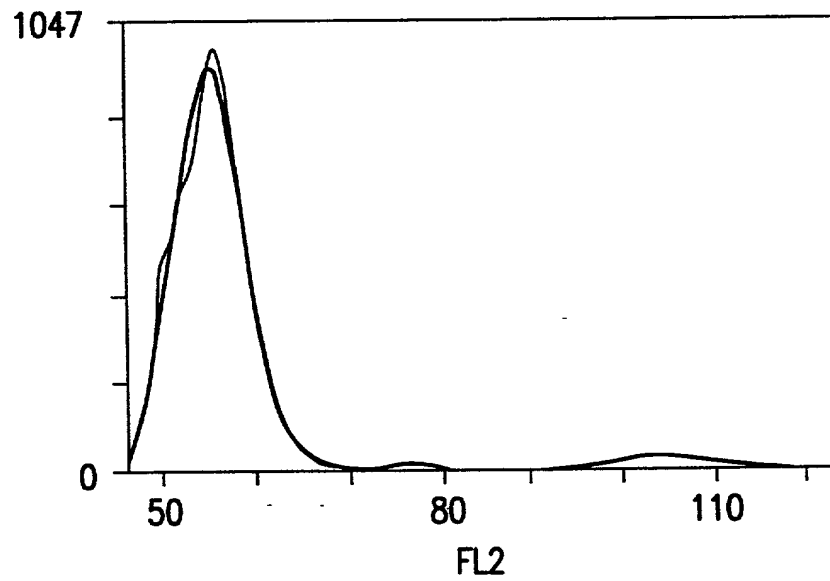


FIG.12D

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FIG.13B



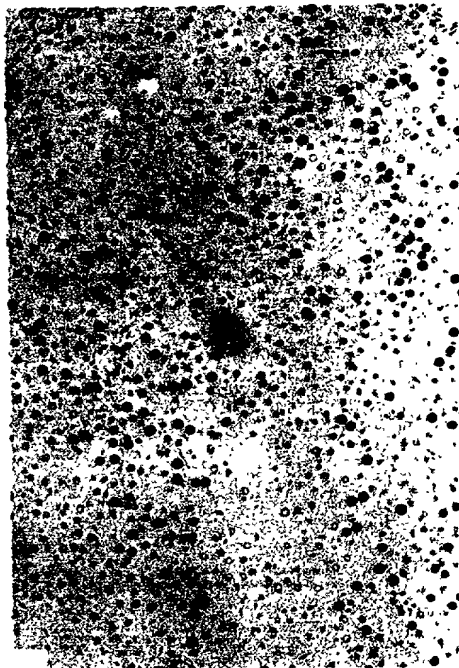
FIG.13D



FIG.13A



FIG.13C



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FIG.14B

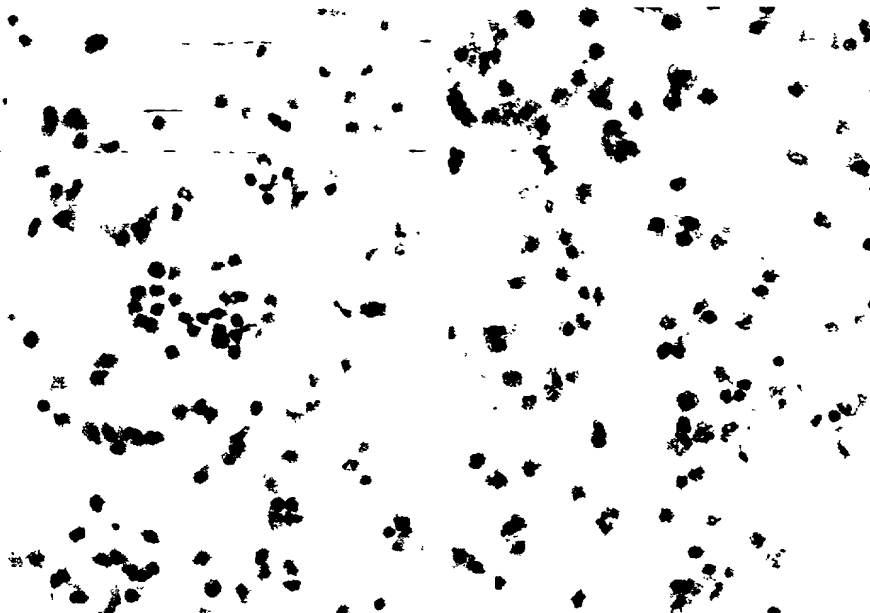


FIG.14D

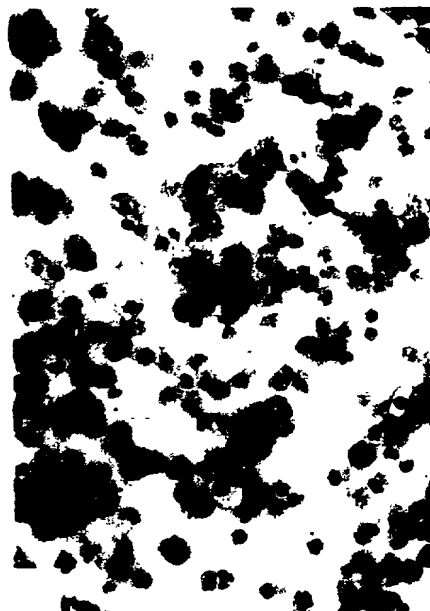
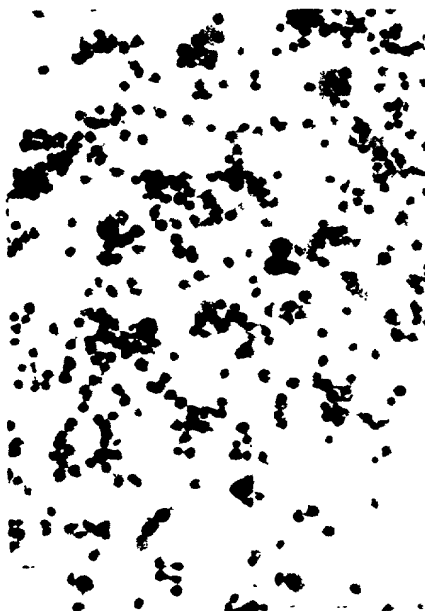


FIG.14C

FIG.14A



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FIG.14F



FIG.14H



FIG.14E

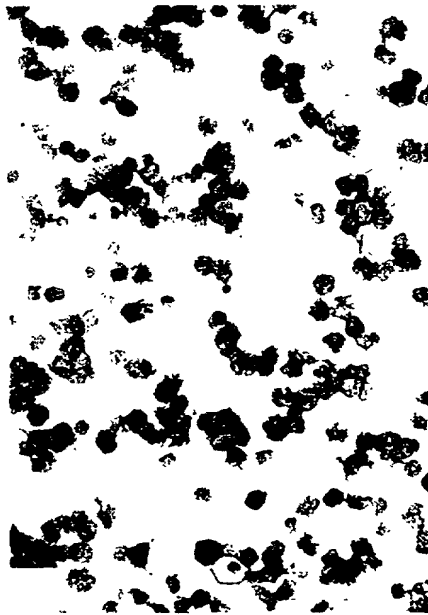


FIG.14G



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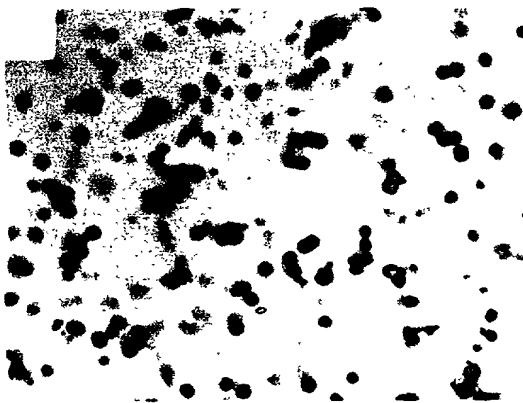


FIG.15A



FIG.15B

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FIG.15C

FIG.15D



FIG.15E

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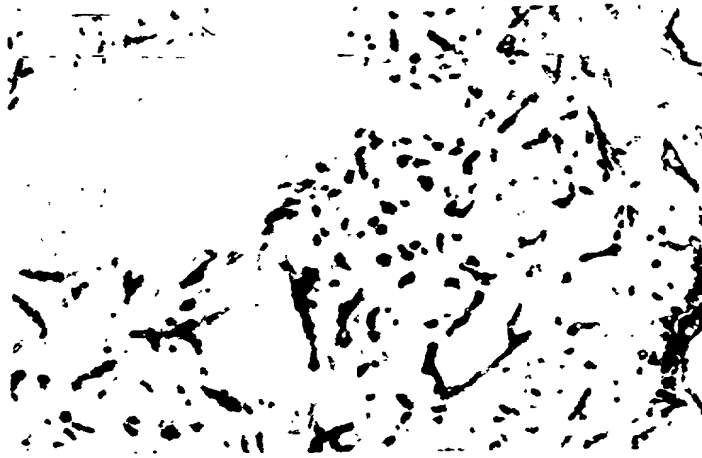


FIG.16A



FIG.16B

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FIG.16C



FIG.16D

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FIG.17A

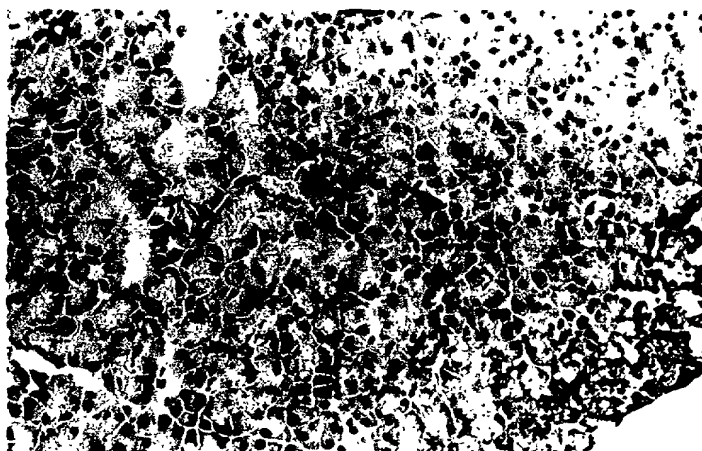


FIG.17B

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FIG.17C



FIG.17D

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FIG.17E

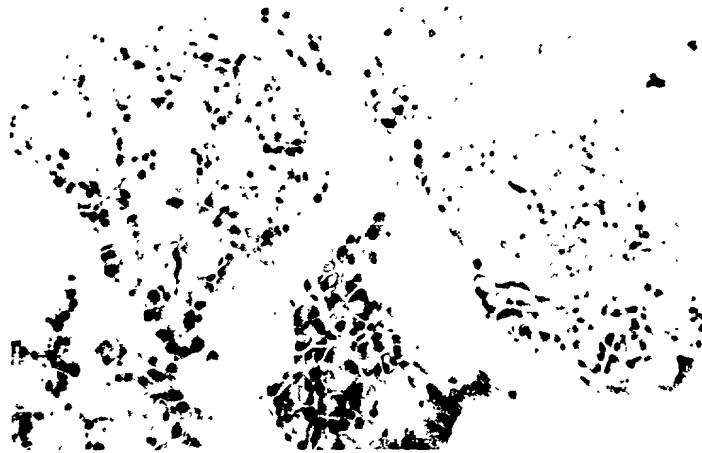


FIG.17F

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**COMBINED DECLARATION
AND POWER OF ATTORNEY**

#20

(Original, Design, National Stage of PCT, Divisional, Continuation or C-I-P Application)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

BONE MARROW TRANSPLANTATION FOR HEPATIC REGENERATION AND REPAIR

This declaration is of the following type:

- ☐ original
- ☐ design
- ☒ national stage of PCT.
- ☐ divisional
- ☐ continuation
- ☐ continuation-in-part (C-I-P)

the specification of which: *(complete (a), (b), or (c))*

- (a) ☐ is attached hereto.
- (b) ☒ was filed on August 24, 2001 as Application Serial No. 09/914,175.
- (c) ☒ was described and claimed in PCT International Application No. PCT/US00/04744 filed on February 25, 2000.

Acknowledgement of Review of Papers and Duty of Candor

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56.

[] In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.98.

Priority Claim

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT International Application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT International Application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application on which priority is claimed

(complete (d) or (e))

- (d) [] no such applications have been filed.
- (e) ☒ such applications have been filed as follows:

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			
COUNTRY	APPLICATION NO.	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
	PCT/US00/04744	February 25, 2000	
ALL FOREIGN APPLICATION[S], IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION			

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date
60/122,250	February 26, 1999

Claim for Benefit of Earlier U.S./PCT Application(s) under 35 U.S.C. 120

(complete this part only if this is a divisional, continuation or C-I-P application)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

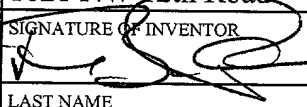
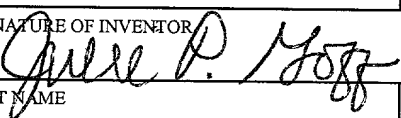
(Application Serial No.)	(Filing Date)	(Status)	(patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status)	(patented, pending, abandoned)

Power of Attorney

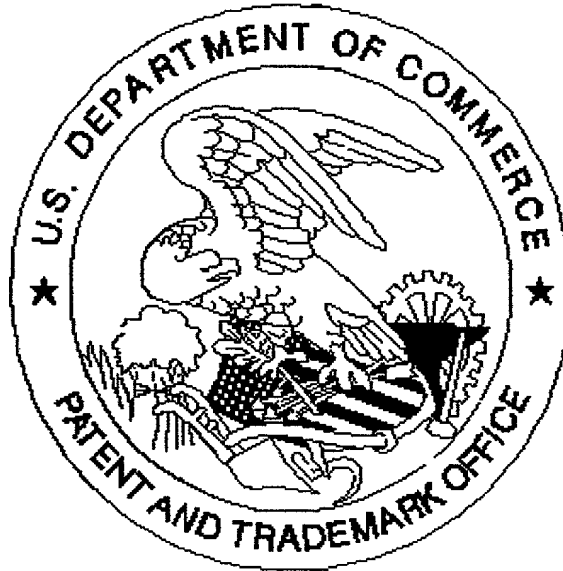
As a named inventor, I hereby appoint Francis J. Hone, Reg. No. 18,662; Joseph D. Garon, Reg. No. 20,420; Arthur S. Tenser, Reg. No. 18,839; Ronald B. Hildreth, Reg. No. 19,498; Thomas R. Nesbitt, Jr., Reg. No. 22,075; Robert Neuner, Reg. No. 24,316; Richard G. Berkley, Reg. No. 25,465; Richard S. Clark, Reg. No. 26,154; Bradley B. Geist, Reg. No. 27,551; James J. Maune, Reg. No. 26,946; John D. Murnane, Reg. No. 29,836; Henry Tang, Reg. No. 29,705; Robert C. Scheinfeld, Reg. No. 31,300; John A. Fogarty, Jr., Reg. No. 22,348; Louis S. Sorell, Reg. No. 32,439; Rochelle K. Seide Reg. No. 32,300; Gary M. Butter, Reg. No. 33,841; and Lisa B. Kole, Reg. No. 35,225 of the firm of BAKER & BOTTS, L.L.P., with offices at 30 Rockefeller Plaza, New York, New York 10112, as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

SEND CORRESPONDENCE TO: BAKER & BOTTS, L.L.P. 30 ROCKEFELLER PLAZA, NEW YORK, N.Y. 10112 CUSTOMER NUMBER: 21003	DIRECT TELEPHONE CALLS TO: BAKER & BOTTS, L.L.P. (212) 705-5000
---	--

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF SOLE OR FIRST INVENTOR		LAST NAME <u>Petersen</u>	FIRST NAME <u>Bryon</u>	MIDDLE NAME <u>E.</u>
RESIDENCE & CITIZENSHIP		CITY <u>Gainesville</u>	STATE or FOREIGN COUNTRY <u>Florida FL</u>	COUNTRY OF CITIZENSHIP <u>United States</u>
POST OFFICE ADDRESS		POST OFFICE ADDRESS <u>1821 NW 12th Road</u>	CITY <u>Gainesville</u>	STATE or COUNTRY <u>Florida</u> ZIP CODE <u>32605</u>
DATE <u>2/5/02</u>		SIGNATURE OF INVENTOR 		
FULL NAME OF SECOND JOINT INVENTOR, IF ANY		LAST NAME <u>Goff</u>	FIRST NAME <u>Julie</u>	MIDDLE NAME <u>P.</u>
RESIDENCE & CITIZENSHIP		CITY <u>Pittsburgh</u>	STATE or FOREIGN COUNTRY <u>Pennsylvania PA</u>	COUNTRY OF CITIZENSHIP <u>United States</u>
POST OFFICE ADDRESS		POST OFFICE ADDRESS <u>7531 Tuscarora Street</u>	CITY <u>Pittsburgh</u>	STATE or COUNTRY <u>Pennsylvania</u> ZIP CODE <u>15208</u>
DATE <u>1/16/02</u>		SIGNATURE OF INVENTOR 		
FULL NAME OF THIRD JOINT INVENTOR, IF ANY		LAST NAME	FIRST NAME	MIDDLE NAME
RESIDENCE & CITIZENSHIP		CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		POST OFFICE ADDRESS	CITY	STATE or COUNTRY ZIP CODE
DATE		SIGNATURE OF INVENTOR		
FULL NAME OF FOURTH JOINT INVENTOR, IF ANY		LAST NAME	FIRST NAME	MIDDLE NAME
RESIDENCE & CITIZENSHIP		CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		POST OFFICE ADDRESS	CITY	STATE or COUNTRY ZIP CODE
DATE		SIGNATURE OF INVENTOR		
FULL NAME OF FIFTH JOINT INVENTOR, IF ANY		LAST NAME	FIRST NAME	MIDDLE NAME
RESIDENCE & CITIZENSHIP		CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		POST OFFICE ADDRESS	CITY	STATE or COUNTRY ZIP CODE
DATE		SIGNATURE OF INVENTOR		
FULL NAME OF SIXTH JOINT INVENTOR, IF ANY		LAST NAME	FIRST NAME	MIDDLE NAME
RESIDENCE & CITIZENSHIP		CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		POST OFFICE ADDRESS	CITY	STATE or COUNTRY ZIP CODE
DATE		SIGNATURE OF INVENTOR		

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